Ghent University
Faculty of Medicine and Health Sciences
Department of Pediatrics and Medical Genetics
2017

Molecular diagnosis of Common Variable Immunodeficiency and related antibody disorders: an integrated immunological and genetic approach

Delfien Bogaert

Promotors
Prof. Dr. Elfride De Baere
Prof. Dr. Filomeen Haerynck
Dr. Melissa Dullaers

Thesis submitted to fulfill the requirements for the degree of
Doctor of Health Sciences

Cover design: Wim Dewitte - Isberg Design Company (www.isberg.be)

ISBN: 9789461975560

Thesis submitted to fulfill the requirements for the degree of Doctor of Health Sciences

2017

Administrative promotor

Prof. Dr. Elfride De Baere
Ghent University, Ghent, Belgium

Co-promotors

Prof. Dr. Filomeen Haerynck
Ghent University, Ghent, Belgium

Dr. Melissa Dullaers
Ghent University, Ghent, Belgium

Examination committee

Prof. Dr. Johan Vande Walle (chair)
Ghent University, Ghent, Belgium

Prof. Dr. Hamida Hammad
Ghent University, Ghent, Belgium

Prof. Dr. Rudi Beyaert
Ghent University, Ghent, Belgium

Prof. Dr. Petra Schelstraete
Ghent University, Ghent, Belgium

Dr. Frauke Coppieters
Ghent University, Ghent, Belgium

Prof. Dr. Michel Moutschen
University of Liège, Liège, Belgium

This work was supported by

Research Foundation Flanders
Opening new horizons

Universitair Ziekenhuis Gent

Center for Primary Immunodeficiencies Ghent

CENTRUM MEDISCHE GENETICA GENT

European Society for Immunodeficiencies

VIB
science meets life

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AFM	asymptomatic family member(s)
ALPS	autoimmune lymphoproliferative syndrome
APDS	activated PI3Kδ syndrome
APRIL	a proliferation inducing ligand
array CGH	microarray-based comparative genomic hybridization
BAFF	B cell activating factor belonging to the TNF family
BAFF-R	B cell activating factor belonging to the TNF family receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
BLK	B-lymphoid tyrosine kinase
bp	base pairs
BTK	Bruton tyrosine kinase
CADD	combined annotation dependent depletion
CCR	CC chemokine receptor
CD	cluster of differentiation
CD40L	CD40 ligand
cDCs	conventional DCs
cDNA	complementary DNA
CID(s)	combined immune deficienc(y)(ies)
CLP	common lymphoid progenitors
CMV	cytomegalovirus
CNVs	copy number variations
CR2	complement receptor 2
CSR	class-switch recombination
cTfh	circulating follicular helper T
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
CVID	common variable immunodeficiency
CXCR	CXC chemokine receptor
DCs	dendritic cells
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EBV	Epstein-Barr virus
ESID	European Society for Immunodeficiencies
FMO	fluorescence minus one

GC	germinal center
gDNA	genomic DNA
GLILD	granulomatous-lymphocytic interstitial lung disease
gnomAD	The Genome Aggregation Database
GTF3A	general transcription factor IIIA
GWAS	genome-wide association stud(y)(ies)
HC	healthy control(s)
HEK	human embryonic kidney
HGUS	hypogammaglobulinaemia of uncertain significance
HLA-DR	human leukocyte antigen-DR
HRCT	high-resolution computed tomography
HSC	hematopoietic stem cells
HSCT	hematopoietic stem cell transplantation
IBD mapping	identity-by-descent mapping
ICON	international consensus
ICOS	inducible T cell co-stimulator
ICOS-L	inducible T cell co-stimulator ligand
IFN	interferon
Ig	immunoglobulin
IgGSD	IgG subclass deficiency
IKZF1	IKAROS family zinc finger protein 1
IL	interleukin
IL-R	interleukin receptor
ILCs	innate lymphoid cells
iNKT	invariant natural killer T
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
IPH	idiopathic primary hypogammaglobulinemia
IRF2BP2	interferon regulatory factor 2 binding protein 2
kb	kilo base pairs
KMT2A	lysine methyltransferase 2A
Leu	leucine
LPS	lipopolysaccharide
LRBA	lipopolysaccharide-responsive beige-like anchor protein
Mb	mega base pairs
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MPP	multipotent progenitors

mRNA	messenger RNA
MS4A1	membrane-spanning 4A1
MSC	mutation significance cutoff
NA	not available
NF-κB	nuclear factor of kappa light chain enhancer of activated B cells
NFAT	nuclear factor of activated T cells
NGS	next-generation sequencing
NK	natural killer
NKT	natural killer T
NMD	nonsense-mediated mRNA decay
no.	number
ns	not significant
PAD(s)	primary antibody deficienc(y)(ies)
PAGID	Pan-American Group for Immunodeficiency
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PHA	phytohemagglutinin
PI3K	phosphatidyl-inositol-3-kinase
PID(s)	primary immunodeficienc(y)(ies)
PIK3CD	PI3K catalytic subunit p110δ
PIK3R1	PI3K regulatory subunit p85α
PKCδ	kinase C delta
PLAID	PLCγ2-associated antibody deficiency and immune dysregulation
PLCG2	phospholipase C gamma 2
PLCγ2	phospholipase C gamma 2
PRKCD	protein kinase C delta
qPCR	quantitative PCR
RAC2	Ras-related C3 botulinum toxin substrate 2
RNA	ribonucleic acid
rRNA	ribosomal RNA
RS	Roifman syndrome
RTE	recent thymic emigrants
SD	standard deviation
SEM	standard error of the mean
SHM	somatic hypermutation

sIgAD	selective IgA deficiency
sIgMD	selective IgM deficiency
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SNP(s)	single nucleotide polymorphism(s)
snRNA	small nuclear RNA
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
TCM	central memory T
TCR	T cell receptor
TEM	effector memory T
TEMRA	effector memory RA T (terminally differentiated T)
Tfh	follicular helper T
TFIIIA	transcription factor IIIA
Th	T helper
TLR(s)	Toll-like receptor(s)
TNF	tumor necrosis factor
TNFRSF	TNF receptor superfamily
TNFSF	TNF superfamily
TPN	total parental nutrition
TREC	T cell receptor excision circle
Treg	regulatory T
tRNA	transfer RNA
TWEAK	TNF-like weak inducer of apoptosis
Tyr	tyrosine
VAV1	Vav1 guanine nucleotide exchange factor
WES	whole exome sequencing
WGS	whole genome sequencing
WSS	Wiedemann-Steiner syndrome
WT	wild type
XLA	X-linked agammaglobulinemia

Chapter 1

Introduction

1 INTRODUCTION

1.1 Antibody-mediated immunity

1.1.1 The immune system at a glance

The immune system is an amazingly elaborate and dynamic system, equipped to protect the body against myriad infectious agents and to eliminate abnormal host cells, without causing undue injury to normal tissues.[1] It is composed of many different organs (e.g. skin, thymus, spleen), tissues (e.g. mucosal membranes, blood and lymphatic vessels, lymph nodes), cell types (e.g. phagocytes, lymphocytes), serum proteins (e.g. complement, antibodies), and signal transduction molecules (e.g. cytokines, receptors, enzymes, transcription factors). While each component has its specific role, all are entangled in a complex network of interactions necessary to orchestrate adequate immune responses.[1]

The immune system is broadly divided in an innate and adaptive arm (Figure 1).[1] **Innate immunity (also called natural immunity)** forms the body's first line of defense against microbes. When epithelial barriers are breached, immune responses are immediately initiated within minutes to hours after invasion (Figure 1).[1] To meet these requirements, components of the innate immune system are mature at birth and are able to recognize a broad range of pathogens in a nonspecific manner.[1] The innate immune response is also important for inducing and directing the adaptive immune response.[1] In contrast to innate immunity, **adaptive or acquired immunity** has an exquisite specificity for distinct microbial substances termed antigens.[1] The disadvantage is that cells of the adaptive immune system need several days to further differentiate and proliferate before reaching a full response (Figure 1).[1] However, after initial exposure to an antigen, the adaptive immune system will have acquired immunological memory providing long-term protection.[1] Upon re-exposure to the same antigen, adaptive immunity will mount a more rapid and potent response enhancing pathogen clearance.[1] Adaptive immune responses are mainly executed by B and T lymphocytes (Figure 1).[1] Of note, B lymphocytes (or B cells) predominantly develop in the *b*one marrow whereas T lymphocytes (or T cells) complete their development in the *t*hymus. During an adaptive immune response, B cells differentiate to produce antigen-specific antibodies, as outlined in the following section (1.1.2).[1] T cells, on the other hand, differentiate into diverse effector cells that perform different functions.[1] $CD4^+$ T helper (Th) cells consist of several subpopulations that either help the activity of other immune cells, such as B cells and phagocytes, or help regulate immune responses.[1] $CD8^+$ cytotoxic T cells kill host cells that are infected, malignantly transformed or damaged in other ways.[1]

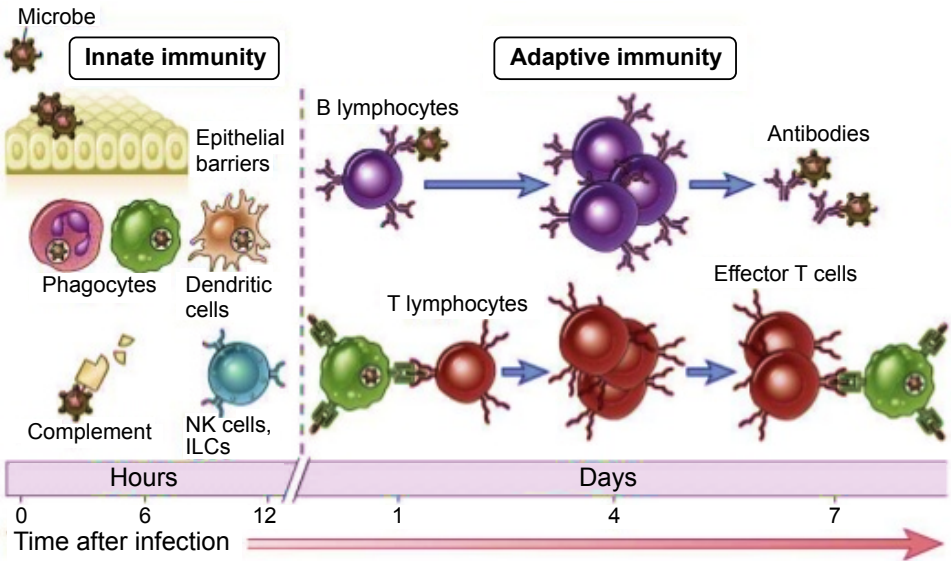


Figure 1. Innate and adaptive immunity. Schematic representation of the main components and kinetics of the innate and adaptive immune responses. ILCs: innate lymphoid cells, NK: natural killer. *Adapted from Abbas AK, Lichtman AH and Pillai S. Cellular and Molecular Immunology. 8th ed. Philadelphia: Elsevier Saunders; 2014.*

In general, immune responses mediated by molecules in extracellular body fluids (called "humors" by the ancient Greeks) are designated as **humoral immunity** (e.g. complement, secreted antibodies). Analogously, cellular components of the immune system provide **cell-mediated immunity** (e.g. phagocytes, T cells).[1] In practice, however, the terminology "humoral immune response" is most often used to refer to the activation of B cells and their differentiation into antibody-secreting factories, and "cell-mediated immunity" is usually ascribed to the actions of T cells.

1.1.2 B cell development and antibody production

1.1.2.1 Introduction

As mentioned above, **antibodies, also known as immunoglobulins**, are produced by cells of the B lymphocyte lineage.[1] Immunoglobulins are composed of two heavy chains and two light chains that together recognize specific antigens.[1,2] They are first expressed on the cell membrane as a B cell antigen receptor (BCR).[1,2] Upon antigen-specific stimulation and differentiation, immunoglobulins are secreted as soluble proteins in blood and mucosal secretions (Figure 1).[1,2] During B cell development and differentiation, immunoglobulins undergo several steps of diversification via somatic rearrangements and point mutations in their germline encoded genes. These diversification steps greatly expand the antibody repertoire and allow the immune system to recognize virtually any antigen.[2,3] To ensure the production of functional non-self-reactive antibodies, B cells progress through a series of well-regulated stages passing both quality control and tolerance checkpoints.[2,4]

1.1.2.2 Antigen-independent B cell development

B cells originate from hematopoietic stem cells, in the liver during fetal life and in the bone marrow after birth (Figure 2).[2] During early B cell development, a **primary repertoire of antibody specificities** is acquired by somatic recombination of immunoglobulin heavy chain and light chain gene segments.[1,2] These recombinations occur randomly and independent of antigen contact.[1,2] **Pro-B cells** first rearrange gene segments of the heavy chain locus (Figure 2).[1,2] The recombined region together with the constant μ and δ gene segments constitutes the primary transcript. Via alternative splicing of this transcript, cells can produce both μ and δ heavy chain proteins. However, expression of δ-chain containing IgD seems to occur predominantly after completion of the early developmental and maturation steps (Figure 2).[2] In the bone marrow, the μ heavy chain and a surrogate light chain form the pre-BCR expressed by the **pre-B cell** (Figure 2).[1,2] Surface expression of the pre-BCR stops further rearrangements of the heavy chain loci, assuring that the developing B cell produces only one antigenic specificity.[2,4]

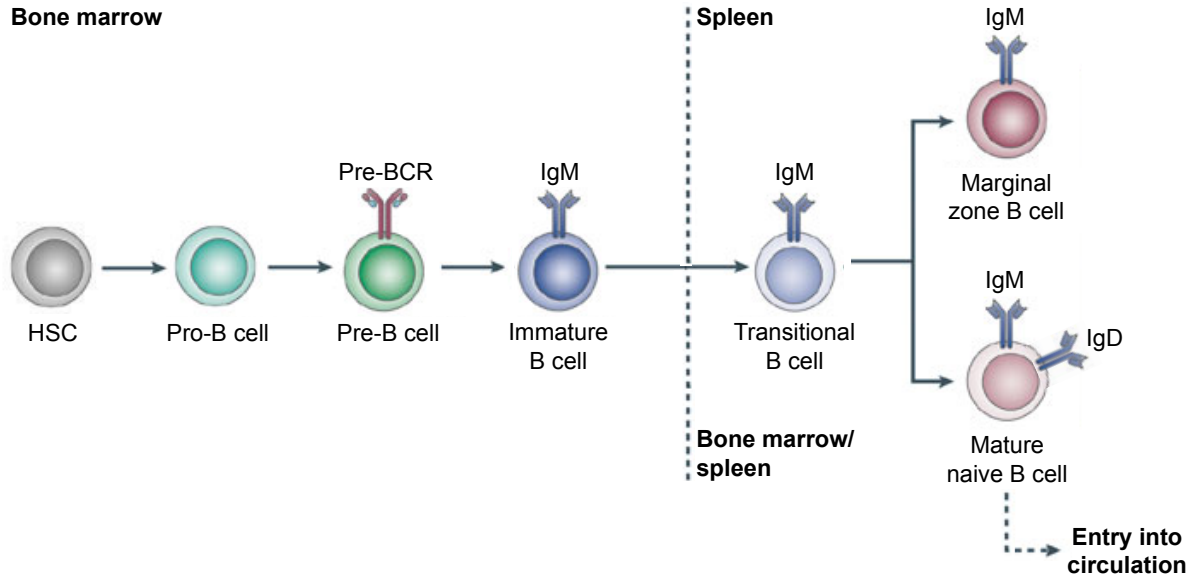


Figure 2. Antigen-independent B cell development. During early B cell development in the bone marrow, precursor B cells only proceed to the next stage after productive rearrangement of the heavy chain (pro- to pre-B cell) and light chain (pre- to immature B cell) immunoglobulin gene segments. The subsequent maturation steps take place in the bone marrow and/or spleen. Mature naive B cells enter the peripheral blood whereas marginal zone B cells reside in the spleen. BCR: B cell receptor, HSC: hematopoietic stem cell, Ig: immunoglobulin. *Adapted from Siebenlist U et al. Nat Rev Immunol. 2005;5(6):435-45.*

Of interest, Bruton tyrosine kinase (BTK) is an important signaling molecule downstream of the pre-BCR and, later, the mature BCR.[2] Mutations in the *BTK* gene, which is located on the X chromosome, cause an early B cell developmental block that results in X-linked

agammaglobulinemia (XLA), a disease first described by Colonel Bruton in 1952 (see section 1.2.1.1).[2,5]

Following heavy chain recombination, pre-B cells recombine gene segments of the κ or λ light chain locus.[1,2] The light chain associates with the previously synthesized μ heavy chain, forming an IgM BCR on the surface of **immature B cells** (Figure 2).[1,2] Note that large numbers of precursor B cells go into apoptosis when somatic recombination does not result in a functional heavy or light chain protein.[4] IgM-expressing immature B cells develop over **transitional B cells** into **mature naive B cells** expressing high levels of both IgM and IgD (Figure 2).[4,6] These maturation steps occur simultaneously in the bone marrow and spleen. A selection of transitional B cells becomes **marginal zone B cells** that reside in the spleen (Figure 2). Mature naive B cells recirculate between the blood and lymphoid organs.

1.1.2.3 Antigen-dependent T cell-dependent B cell development

Typically, mature naive B cells recirculate through peripheral lymphoid organs until they encounter their specific antigen (Figure 3).[2,7] Binding of antigen to its specific BCR triggers B cell activation.[1] The antigen is then internalized, processed and presented on the B cell surface via major histocompatibility complex class II (MHC II) molecules, where it can be recognized by cognate T cells.[1] Cognate naive $CD4^+$ Th cells are first activated upon recognition of the same protein antigen presented by **dendritic cells (DCs)** via MHC II (Figure 3).[1] Next, initial cognate interactions between activated B cells and Th cells take place at the border between B cell follicles and T cell zones (Figure 3).[7,8] After these initial interactions, activated B cells can migrate to the extrafollicular areas of peripheral lymphoid organs where they proliferate and terminally differentiate into short-lived plasma cells (Figure 3).[7,8] Alternatively, activated B cells can move into B cell follicles and engage in what is known as the **germinal center (GC) reaction** (Figure 3).[2,7,8]

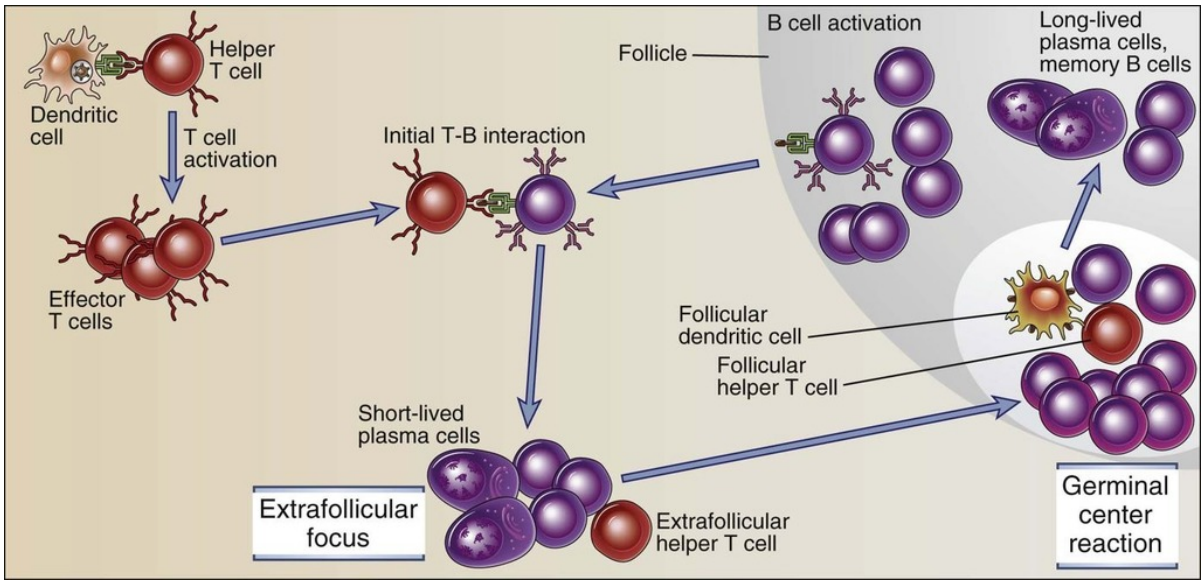


Figure 3. T cell-dependent antibody responses in peripheral lymphoid organs. Upon encounter of a protein antigen presented by dendritic cells in peripheral lymphoid tissues, B cells are activated with help of T cells. After the initial T-B interaction, B cells can move to extrafollicular areas and differentiate into short-lived plasma cells with limited affinity maturation and class switching. Alternatively, B cells can migrate into B cell follicles and engage in a germinal center reaction with affinity maturation, class switching and terminal differentiation into memory B cells and long-lived plasma cells. *Adapted from Abbas AK, Lichtman AH and Pillai S. Cellular and Molecular Immunology. 8th ed. Philadelphia: Elsevier Saunders; 2014.*

The formation of GCs requires intense reciprocal interactions between cognate B cells and **follicular Th (Tfh) cells**, of which the most important are shown in Figure 4.[2,7,8] These interactions provide B cells with the necessary helper signals for proliferation and differentiation, and also enhance the effector functions of Tfh cells.[2,7,8]

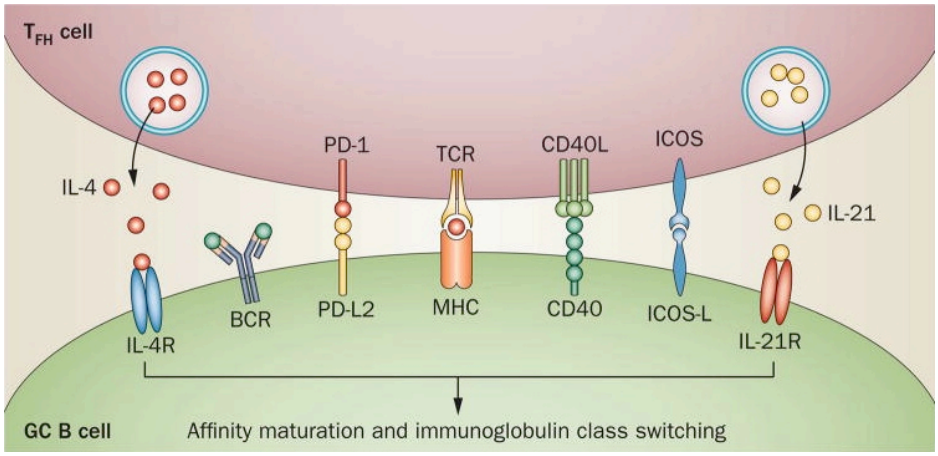


Figure 4. Reciprocal interactions between follicular helper T (T_{FH}) cells and germinal center (GC) B cells. T_{FH} cells promote survival, proliferation and terminal differentiation of B cells into memory B cells and class-switched affinity-maturated antibody-secreting plasma cells. In turn, these costimulatory signals enhance T_{FH} cell effector functions. BCR: B-cell receptor, CD40L: CD40 ligand, ICOS(-L): inducible T cell co-stimulator (ligand), IL-4(R): interleukin 4 (receptor), IL-21(R): interleukin 21 (receptor), MHC: major histocompatibility complex, PD-1, programmed death-1, PD-L2: programmed death ligand 2, TCR: T-cell receptor. *Adapted from Craft EJ. Nat Rev Rheumatol. 2012; 8(6): 337-47.*

GCs are the primary sites for **somatic hypermutation (SHM)**, **class-switch recombination (CSR)**, and **terminal differentiation** of B cells into **antibody-secreting plasma cells** and **memory B cells** (Figure 3).[1,6,8] SHM introduces point mutations at high rates in the heavy chain and light chain regions important for antigen recognition and binding.[1,2,8] This process expands the diversity of the antibody repertoire (**secondary repertoire**) and allows for the production of higher-affinity antibodies against the pathogen at hand.[8] For that reason, SHM is also called affinity maturation.[8] During SHM, B cells undergo several cycles of clonal deletion and selection to eliminate B cells with lower-affinity or autoreactive BCRs.[1,2,4,8] Immunoglobulin classes or isotypes, and their related effector functions, are determined by the constant regions of the heavy chain.[1,7,8] CSR or isotype switching is the process that changes the B cell's IgM/IgD co-production to a single isotype production (IgG, IgA, IgE, IgM or IgD), via somatic gene rearrangements of the heavy chain constant regions.[1,6,8] CSR does not alter antigen specificity.[8] Following SHM and CSR, B cells terminally differentiate into either memory B cells or long-lived antibody-secreting plasma cells (Figure 3).[8] Memory B cells are long-lived and can rapidly differentiate into high-affinity antibody-secreting plasma cells upon re-exposure to the same antigen.[7] Long-lived plasma cells migrate primarily to the bone marrow where they can reside for years.[7] These plasma cells provide long-term antibody titers but do not self-renew.[7] Together, memory B cells and long-lived high-affinity antibody-secreting plasma cells ensure sustained humoral immune protection.[7]

1.1.2.4 Antigen-dependent T cell-independent B cell development

The T cell-dependent antibody responses outlined above are only initiated after exposure to protein antigens.[1,6] Hence, proteins are classified as **T-dependent antigens**.[1,6] In contrast, nonprotein antigens such as polysaccharides, glycolipids and nucleic acids, cannot be presented to T cells in the context of MHC II molecules.[1,6] They elicit T cell-independent antibody responses and are, therefore, classified as **T-independent antigens**.[1,6]

In the absence of T cell help, B cells can be activated by different mechanisms.[2,6] Some nonprotein antigens (e.g. bacterial capsular polysaccharides) contain multiple identical epitopes that facilitate BCR oligomerization and subsequent B cell activation.[1,6] Other nonprotein antigens (e.g. certain nucleic acids) promote B cell activation by engaging so-called Toll-like receptors (TLRs) (see also sections 1.5.2.5 and 1.6.2.1 and Figure 16).[1,2,6] In addition, activated DCs can secrete the costimulatory molecules B cell activating factor belonging to the TNF family (BAFF) and a proliferation inducing ligand (APRIL) that bind a common receptor on B cells known as transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) (see also Figure 16).[6,9] TACI signaling is believed to trigger T cell-independent isotype switching, thereby enhancing antibody responses to

nonprotein antigens.[2,6,10] The functions of BAFF, APRIL and TACI will be discussed in more depth in section 1.6.2.1, paragraph “TACI and BAFF-R”.

T cell-independent antibody responses are predominantly mediated by marginal zone B cells in the spleen (Figure 2) and B cells residing in mucosa-associated lymphoid tissues.[2,6] Antibodies produced in the absence of T cell help are of lower affinity and consist mainly of IgM with limited isotype switching.[1,2,6] However, T cell-independent antibody responses can be mounted more quickly, already providing protection during the first days of infection (Figure 1).[2,6]

1.1.3 The role of antibodies in host defense

Antibodies present in the blood and mucosal secretions are indispensable in host defense against extracellular bacteria, fungi and microbial toxins.[1,11] Antibodies are also involved in immune responses against obligate intracellular microbes such as viruses, which can be targeted before they infect cells or when they are released from infected cells.[1,11] Of note, T cell-independent antibody responses mounted by marginal zone B cells in the spleen constitute the first line of defense against blood-borne pathogens, and are particularly important for defense against polysaccharide encapsulated bacteria (see section 1.1.2.4).[2,6]

Secreted antibodies provide protection by performing three main effector functions (Figure 5).[11] First, antibodies can **neutralize** the pathogenic effects of microbes and microbial toxins by blocking key sites for binding to cellular receptors.[11] Second, antibodies can coat the surface of microbes to enhance their elimination by phagocytosis, a process referred to as **opsonization**.[11] Third, antibodies coating a microbe can also trigger **activation of complement proteins** that, in turn, promote opsonization, recruitment of phagocytes and lysis of certain bacteria.[11]

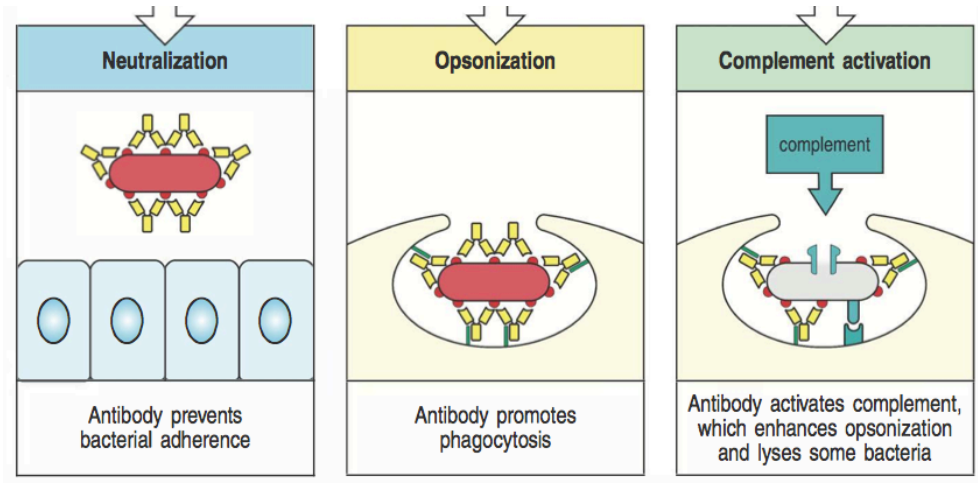


Figure 5. The main effector functions of secreted antibodies. *Adapted from Murphy K. Janeway’s Immunobiology. 8th ed. New York: Garland Science, Taylor & Francis Group, LLC; 2012.*

1.2 Inborn errors of the immune system

1.2.1 Primary immunodeficiencies

Primary immunodeficiencies (PIDs) are a heterogeneous group of disorders caused by genetically determined defects of the immune system.[12] To date, more than 300 monogenic (i.e. single-gene) inborn errors of immunity have been described.[12,13]

1.2.1.1 Historical perspectives

When looking back, progress in the field of PID has always been driven by advances in science and medicine and the development of new technologies.[14] The history of PIDs starts in 1952 with the discovery of X-linked agammaglobulinemia (XLA) by Ogden Bruton (see section 1.1.2.2).[5] Yet, the groundwork had already been laid by 19th century pioneering scientists who introduced innovative ideas in pathology, microbiology, biochemistry, hematology and immunology.[14] Before World War II, a few patients had been reported with typical clinical presentations that would be categorized as PIDs years later. Still, physicians and scientists at that time did not grasp the concept of immunodeficiency.[14] In the late 1940's, advances in public sanitation and hygiene and the advent of antibiotics lowered the global burden of infectious diseases, thereby paving the last part of the way towards the recognition of PIDs.[14] Finally, by using a novel electrophoresis apparatus that had just become available, Bruton was able to confirm his speculation that the recurrent pneumonias of the XLA patient were linked to abnormalities in serum antibodies.[14] In the following decades, only a handful of new PID entities were described.[14] However, in that same period, groundbreaking progress was taking place in molecular genetics: discovery of the DNA double helix structure by Watson and Crick in 1953, deciphering of the genetic code and mechanisms of DNA replication in the 1950s and 60s, development of Sanger sequencing and PCR in the 1970s and 80s, and the launch of the Human Genome Project in 1990.[14] In the mid 1990s, encouraged by the possibilities of gene sequencing and increasing insights in basic immunology, physicians and scientists showed a renewed interest in PIDs.[14,15] From then on, a multitude of novel PID diseases and genes were being reported at a continual pace.[15] Since 2010, the field of PID has, again, been transformed by the advent of next-generation sequencing (NGS) technologies.[15] NGS technologies have facilitated the genetic diagnosis of PID patients and the identification of novel PID-causing genes.[15] Even more, this most recent surge is substantially enhancing our understanding of disease pathophysiology as well as basic cellular pathways and mechanisms of host defense.[16] Considering that whole genome sequencing, genome editing technologies and functional methods keep on improving, we expect that research efforts in the field of PID will continue to grow for years to come.[15-17]

1.2.1.2 Epidemiology

Individual PID disorders are very rare, but collectively they pose a health burden that is believed to be comparable with that of cystic fibrosis or leukemia.[18,19] Despite enormous efforts, patient registration remains, however, incomplete and inconsistent across continents. Additionally, it is suspected that a considerable number of PID patients are undiagnosed. Estimates of the prevalence of PIDs from registry data worldwide range from 5.3 to 86.3 per 100,000, with an estimated incidence of up to 10.3/100,000 per year.[19] Despite the lack of accurate prevalence and incidence rates, PIDs seem to be more frequent than originally thought.[19] Some authors state that the prevalence of PIDs may be as high as 1-2% of the population when taking into account all types and varieties.[13] With the exception of X-linked conditions, PIDs seem to affect men and women equally.[13] The age of onset is very variable.[19] In general, the more severe the PID condition, the earlier the age of onset.[20] More than 90% of PID patients registered worldwide were diagnosed under the age of 15 years.[19] However, an important bias to be considered is that registration is mainly done by pediatric centers.[19] Although exact numbers are not available, patient registries as well as individual case reports clearly indicate that PIDs are not exclusively diseases of infancy or early childhood but can manifest at any age up to late adulthood.[13,19]

1.2.1.3 Clinical phenotypes

The genetic defects underlying PID diseases result in reduced expression and/or aberrant function of one or more components of the immune system.[12] Accordingly, each PID disease is characterized by its own palette of clinical and immunological (laboratory) abnormalities.[12,20] Some PIDs are severe or even life threatening, whereas others are mild and may go unnoticed for years.[12] Notwithstanding this heterogeneity, the clinical features seen in PIDs can be categorized into **five major phenotypes** (i.e. physical characteristics) as shown in Figure 6.[20] PID patients commonly have an increased susceptibility to **infections**.[12,20] These infections are recurrent, severe, persistent, complicated and/or caused by unusual (opportunistic) organisms.[12,20] The type of infection can sometimes provide a clue in which part of the body's defense mechanisms the immune defect is situated.[20] Infections are, however, not the only health problem faced by PID patients. Errors of immunity often cause an imbalance between the countless activating, inhibiting and regulating forces that are continually at play. This imbalance creates a situation of **immune dysregulation** that is reflected in **autoimmunity**, **(auto)inflammation**, and/or **allergy**.[12,20,21] Finally, PID patients often have an increased risk of **malignancies**.[12,20,22] This is attributed to aberrant immune surveillance resulting from defective elimination of abnormal or "transformed" host cells and/or defective immune responses against cancer cells.[23]

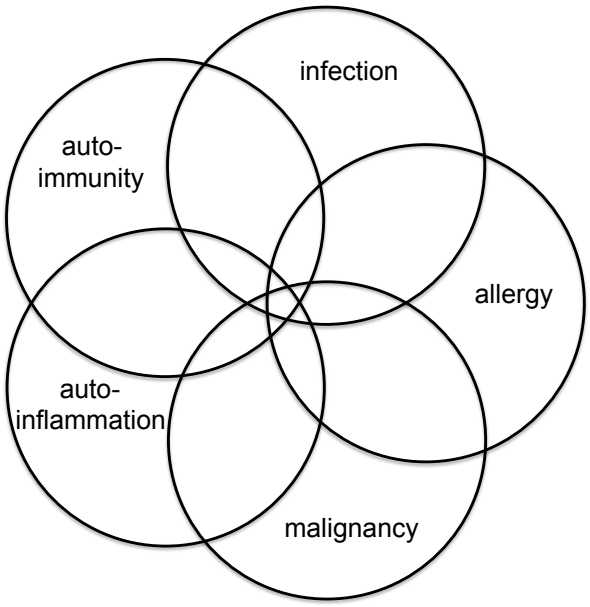


Figure 6. Venn diagram illustrating the variety of clinical phenotypes that underlie primary immunodeficiencies (PIDs). PID patients commonly present with recurrent, severe and/or unusual infections. In addition, they can demonstrate any combination of features related to autoimmunity, (auto)inflammation, allergy and/or malignancy.

1.2.1.4 Monogenic but not always Mendelian

Monogenic inborn errors of immunity can be inherited in an autosomal dominant, autosomal recessive or X-linked recessive manner, or can appear *de novo*.[12,15] The molecular dissection of PID patients over the past two decades has made it clear that the disease phenotype is often not predictive of the underlying genotype (i.e. genetic defect) and *vice versa*.[12,15] A single phenotype can, for example, be caused by mutations in different genes; a situation referred to as **locus heterogeneity**.[15,20,24] Furthermore, different mutations in a single gene can underlie a spectrum of phenotypes with variable severity and age of onset (**allelic heterogeneity**).[12,15,24] For the latter, a genotype-phenotype correlation is rarely observed.[12,15,20] Moreover, the same mutant genotype can also be associated with large phenotypical variability (**variable expressivity**).[12] In addition, a growing number of PID-causing genes are associated with **reduced or incomplete penetrance**, meaning that not all individuals with a mutant genotype develop a phenotype.[15,24] Indeed, it is becoming increasingly evident that PIDs do not always obey the rules of Mendelian inheritance.[25] Current understanding on the molecular basis of variable expressivity and incomplete penetrance is limited.[26] They may be partially explained by the actions of modifier genes, epigenetic changes and/or environmental exposures such as pathogens and immunomodulatory treatments.[15,26] Finally, in some PIDs the affected gene is more widely expressed in human tissues and involved in more universal cellular processes rather than restricted to the immune system. This explains why some patients also demonstrate extra-

immunological manifestations (e.g. skeletal or dental abnormalities); a phenomenon termed **pleiotropy**.[15,27] Although many PIDs can be accompanied by some form of extra-immunological manifestations, disorders are only referred to as syndromic PIDs when these extra-immunological abnormalities are prominent.
The here-mentioned genetic concepts will be further illustrated in section 1.6.

1.2.2 Primary antibody deficiencies

PIDs are classified into different groups in accordance with their most pronounced and fundamental defect.[12] **Primary antibody deficiencies** (or **predominantly antibody deficiencies**) (**PADs**) are the most common group of PIDs, accounting for 50 to 60% of all registered patients.[13,22] PADs are characterized by markedly reduced serum levels of one or more immunoglobulin isotypes and/or impaired antibody responses to specific antigens due to genetically determined defects in B cell development and/or function (see section 1.1.2), without major impairments in other parts of the immune system.[12,28] Disorders affecting other parts of the immune system (e.g. T cells) can also be accompanied by impaired antibody production (see section 1.1.2), but these are usually classified in other PID groups since antibody deficiency is not their most fundamental defect.[12]

The degree of antibody deficiency differs across the various types of PADs.[12,20,28] The prototype disorder of PADs is Bruton's XLA caused by mutations in the *BTK* gene (see sections 1.1.2.2 and 1.2.1.1).[5,28] The block in early B cell development is typically associated with very low to absent B cells and a profound decrease in all serum immunoglobulin isotypes.[28] In other PADs, B cell development and/or function may be less severely affected, causing, for example, selective deficiencies in IgA, IgG subclasses, or antibody responses to specific antigens.[12,20,28] Furthermore, the degree of antibody deficiency can sometimes evolve over time, either improving or getting worse.[6,29] A well-known example is transient hypogammaglobulinemia of infancy, in which serum immunoglobulin levels spontaneously correct by the age of two to four years.[6,30] It is believed that transient antibody deficiencies in young children represent a physiological delay in the maturation of the immune system.[30,31] In other cases, the antibody deficiency may only partially improve or, in contrast, aggravate over time with or without aggravation in additional parts of the immune system.[6,29,32] The underlying mechanisms of these phenomena remain, thus far, enigmatic.

Common variable immunodeficiency (CVID), the focus of this thesis, is one of the most frequently diagnosed PADs.[13,29] Like for all PIDs, there are no precise data on the prevalence of CVID (see section 1.2.1.2).[29] Prevalence estimates range from 1/100,000 to 1/10,000.[33]

The diagnostic, clinical and pathophysiological aspects of CVID will be reviewed in the following sections.

1.3 CVID, a diagnosis of exclusion

The term “common variable immunodeficiency” (CVID) was first introduced in 1971 by a committee of the World Health Organization.[34] This new term had to distinguish less well-defined forms of antibody deficiency from others that had a more coherent clinical picture and Mendelian inheritance such as XLA.[29,34] So, from the start, CVID became a diagnosis of exclusion.[29,34] In need of a clearer definition, the European Society for Immunodeficiencies (**ESID**) and Pan-American Group for Immunodeficiency (**PAGID**) formulated the **first consensus diagnostic criteria of CVID in 1999**:[35]

Probable CVID

Male or female patient who has a marked decrease (at least 2 standard deviations below the mean for age) in serum IgG and in at least one of the isotypes IgM or IgA, and fulfills all of the following criteria:

1. Onset of immunodeficiency at greater than 2 years of age.
2. Absent isohemagglutinins and/or poor response to vaccines.
3. Defined causes of hypogammaglobulinemia have been excluded according to a list of differential diagnoses (Table 1).

Possible CVID

Male or female patient who has a marked decrease (at least 2 standard deviations below the mean for age) in one of the major isotypes (IgM, IgG, and IgA) and fulfills all of the following criteria:

1. Onset of immunodeficiency at greater than 2 years of age.
2. Absent isohemagglutinins and/or poor response to vaccines.
3. Defined causes of hypogammaglobulinemia have been excluded according to a list of differential diagnoses (Table 1).

The first criterion (onset over 2 years of age) mainly served to separate CVID from transient hypogammaglobulinemia of infancy (see section 1.2.2). The third criterion excluded secondary (acquired) causes of antibody deficiency as well as other primary (inborn) causes such as chromosomal disorders and distinct PID disorders (Table 1).

Table 1. Differential diagnoses of hypogammaglobulinemia (non-exhaustive list).

Drug induced
Glucocorticoids, rituximab, captopril, carbamazepine, fenclofenac, penicillamine, phenytoin, sulfasalazine, gold salts, antimalarial drugs
Single gene and other defects
Ataxia telangiectasia, severe combined immunodeficiency (SCID) and other forms of combined immunodeficiency (CID), hyper-IgM syndromes, X-linked agammaglobulinemia, X-linked lymphoproliferative disorder, transcobalamin II deficiency, some metabolic disorders
Chromosomal anomalies
Trisomy 21, 18q deletion syndrome, monosomy 22, trisomy 8
Infectious diseases
Human immunodeficiency virus, Epstein-Barr virus, cytomegalovirus, measles virus, congenital infection with rubella virus or *Toxoplasma gondii*
Malignancy
Chronic lymphocytic leukemia, thymoma, non-Hodgkin lymphoma, monoclonal gammopathy (multiple myeloma, Waldenstrom macroglobulinemia)
Other systemic disorders
Immunodeficiency caused by excessive loss of immunoglobulins (nephrotic syndrome, severe burns, lymphangiectasia, protein-losing enteropathy)

Adapted from Bonhilla et al. J Allergy Clin Immunol Pract. 2016;4(1):38-59.

The ESID/PAGID 1999 criteria stood firm for more than a decade, but with the expanding knowledge on PIDs there was a growing appreciation that the definition of CVID needed to be further refined.[35] The main stumbling block was that the ESID/PAGID 1999 criteria did not always allow to discriminate CVID from overlapping PID disorders, particularly less severe forms of combined immune deficiencies (CIDs; i.e. associated with both B and T cell defects).[36] Between 2013 and 2015, three different revisions of the ESID/PAGID 1999 criteria were proposed.[37-39] In **2013**, **Ameratunga *et al.*** suggested a diagnostic algorithm with limited major and multiple minor (supportive) criteria including various clinical, laboratory and histological features associated with CVID (see sections 1.4 and 1.5).[37] Remarkably, neither IgA nor IgM was required to be low as only a reduction in serum IgG was incorporated in the major criteria.[37] Furthermore, the authors introduced an additional category termed **"hypogammaglobulinaemia of uncertain significance" (HGUS)** for patients that did not meet any of the minor criteria.[37] In **2014**, **ESID** revised the criteria used for their online patient registration database, including the CVID criteria.[38] Similar to the Ameratunga 2013 criteria, the new ESID criteria encompassed the presence of clinical symptoms or a positive family history, an age at diagnosis above 4 years (although symptoms may be present before), and reduced switched memory B cells (see section 1.5.2) as an alternative criterion for poor vaccine responses.[38] However, in contrast to the Ameratunga 2013 criteria, the revised ESID registry 2014 criteria also included the mandatory decrease of serum IgA and defined the exclusion of patients with profound T cell deficiency to facilitate distinction with CIDs.[38] Finally, in **2015**, a French group (**DEFI**) supported the mandatory decrease of IgA,

did not include poor vaccine responses and proposed more restrictive criteria for the exclusion of patients with T cell defects (occurrence of opportunistic infections and very low naive CD4+ T cells).[39] Additionally, they included a category termed "**idiopathic primary hypogammaglobulinemia (IPH)** / HGUS", reminiscent of the HGUS category by Ameratunga *et al.*[37,39] However meritorious the three revisions were, the diagnostic criteria by Ameratunga *et al.*[37] did not seem to find broad acceptance in the immunological community, and not all clinical immunologists agreed on the obligatory decrease in IgA incorporated in the revised ESID registry 2014[38] and DEFI 2015[39] criteria.

In **2016**, an expert committee published a **new international consensus (ICON) definition of CVID**, stating that all of the following criteria should be fulfilled:[29]

1. Decrease in serum IgG according to the age-adjusted reference range. The IgG level must be repeatedly low in at least 2 measurements more than 3 weeks apart. Repeated measurement may be omitted if the level is very low (<100-300 mg/dL depending on age).
2. Decrease in serum IgA and/or IgM (low IgA preferred).
3. If IgG >100 mg/dL: demonstrable impairment of response to at least one protein and/or polysaccharide vaccine.
4. Other causes of hypogammaglobulinemia must be excluded, with special attention for CID characterized by T cell deficiency (Table 1).

The expert committee strongly recommended not to confer a diagnosis of CVID to patients younger than 4 years of age. Furthermore, they provided supportive information how antibody responses to vaccines should be assessed. Of note, characteristic clinical manifestations (infection, autoimmunity, lymphoproliferation) can support a diagnosis of CVID but are not required.

The definition of CVID is still a topic of ongoing debate.[29,40] The latest CVID ICON 2016 diagnostic criteria closely resemble the original ESID/PAGID 1999 criteria, although there is no longer a distinction between a "probable" and "possible" diagnosis of CVID.[29,35] The biggest challenge is how to classify patients presenting clinical features reminiscent of CVID but with only a decrease in one major immunoglobulin class (IgG, IgM, IgA) or an IgG subclass (IgG1-4). The Ameratunga 2013 and DEFI 2015 criteria tried to meet that challenge by introducing an additional category in their diagnostic flow charts, which they termed HGUS or IPH.[37,39] Other expressions used in literature to describe these patients are unspecified/unclassified hypogammaglobulinemia[29,41] or CVID-like disorders[42]. Despite the lack of a clear definition or name, others and we have provided evidence that these milder

forms of antibody deficiency might be part of the same disease spectrum as CVID (see Chapter 3).[6,29,32,43-45]

1.4 Clinical characteristics of CVID

Like most diagnoses of exclusion, CVID encompasses a heterogeneous patient population demonstrating a diversity of clinical and immunological (see section 1.5) manifestations with varying severity.[46-48] Disease onset can occur at any age from infancy to late adulthood.[48-51] Two large cohort studies (n=248, n=2212) reported a peak incidence in the first and third decades of life.[48,49] Approximately 25% of CVID patients receive a diagnosis in childhood.[50]

CVID patients commonly suffer from **recurrent infections**, mainly of the respiratory and gastrointestinal tracts.[48,51] Typical pathogens are encapsulated bacteria (e.g. *Streptococcus pneumoniae*), nontypeable *Haemophilus influenzae*, *Enterobacteriaceae*, *Campylobacter* spp., *Giardia lamblia*, *Mycoplasma* spp., and respiratory viruses.[6,52] This reflects the important role of antibodies in host defense against these organisms (see section 1.1.3). Repeated infections of the lower airways may cause structural damage such as bronchiectasis.[48,53]

Depending on the study cohort, between 33% and 80% of CVID patients have an "infections-only" phenotype.[51,54,55] Other patients additionally develop complications due to immune dysregulation: autoimmunity, enteropathy, polyclonal lymphoproliferation, granulomatous disease, and/or malignancy.[47,48,51,53] These so-called **noninfectious or disease-related complications** can develop any time during the disease course. They can also be the first clinical presentation of disease before the onset of infections.[47,53] Their presence is usually associated with abnormalities in B and/or T cell subsets, which will be explained in the following section (1.5). The pathogenesis of noninfectious complications in CVID remains, however, incompletely understood.[56,57] **Autoimmunity** is seen in up to 25-30% of CVID patients and can affect any part of the body.[48,53,57] Most frequent are autoimmune cytopenias, especially immune thrombocytopenic purpura and autoimmune hemolytic anemia.[48,53] Other autoimmune diseases often encountered in CVID patients include pernicious anemia, thyroid disease, arthritis, vitiligo and systemic lupus erythematosus (SLE).[48,57] **Unexplained enteropathy** with chronic diarrhea and malabsorption is a recurring feature in CVID.[48] The cause of the chronic intestinal inflammation is unclear.[33] In some cases, it may be associated with autoimmunity.[33] At least one third of patients with CVID show signs of **polyclonal lymphoproliferation**.[48,53] Generalized benign lymphadenopathy and splenomegaly are most frequently observed.[48,53] Lymphocytes can also infiltrate other organs such as the liver, gut, brain and lungs.[48,53] Lung infiltration may cause lymphocytic interstitial pneumonitis (LIP).[48,53] Another characteristic complication of CVID is the development of **granulomatous disease**.

Granulomas are typically seen in the lungs, but virtually any organ can be involved.[48,53] The co-occurrence of granulomas and lymphoid infiltrates in the lungs is referred to as granulomatous-lymphocytic interstitial lung disease (GLILD).[33] About 6-9% of CVID patients are diagnosed with **malignancy**, mostly lymphoma.[56] Patients also have an increased risk for solid tumors, particularly gastric cancer.[29,33]

1.5 Immunological abnormalities in CVID

1.5.1 Introduction

The hallmark immunological defect in CVID patients is, per definition, impaired immunoglobulin production by plasma cells resulting in hypogammaglobulinemia and poor vaccine responses.[29] Besides defects in the B cell lineage, numerous abnormalities in the T cell and innate immune compartment have been reported.[46] The broad distribution of defects supports the concept of CVID being a condition of generalized immune dysregulation rather than an isolated antibody deficiency.[46]

The connection between B cell, T cell and innate immune cell abnormalities in disease development remains largely enigmatic. The various immunological abnormalities reported in the CVID population are unequally distributed across patient cohorts. Therefore, no coherent picture can be drawn from the myriad of immunophenotypical and functional studies performed in the past decades. Furthermore, it is difficult to determine whether immunological defects are primary (disease-intrinsic) or secondary (e.g. caused by chronic inflammation), especially because the underlying pathophysiological mechanisms are mostly unknown and likely to be heterogeneous.[29,46]

1.5.2 B cell abnormalities

In keeping with primary antibody failure as the hallmark of CVID, the B cell compartment has been the main subject of research. This section elaborates on the most important numerical and functional B cell defects described in CVID patients thus far.

1.5.2.1 Total peripheral B cell numbers

Total peripheral B cell numbers are variable. In more than half of patients, B cell numbers are within normal ranges for age.[49,53,58-60] About 10% has reduced total B cells, and in 5-10% of patients B cells constitute less than 1% of total peripheral lymphocytes.[49,53,58-60] In contrast, depending on the study cohort, up to 20% of CVID patients have increased levels of total B cells.[49,53,58-60]

Low or absent B cells indicates a defect in B cell development and/or B cell survival.[46,60] Some CVID patients with low B cell counts were found to have a partial ***block in early B cell development*** or a decrease in hematopoietic precursor cells in the bone marrow (see also section 1.1.2.2).[61,62] The authors postulated that this could result from a primary defect in bone marrow progenitor cells or from damage caused by an altered cytokine environment secondary to chronic or recurrent infections.[61,62] The latter hypothesis has, to our knowledge, not yet been investigated. Still, in most patients with decreased peripheral B cells, bone marrow development appeared to be normal.[61] Also, some patients with low B cells have a normal peripheral B cell differentiation pattern. These findings suggest that other factors are involved in peripheral B cell depletion, which may ***reduce survival*** and/or ***promote apoptosis***.[61] Survival of circulating B cells is dependent on BCR and BAFF-receptor (BAFF-R) signaling (see also section1.6.2.1, paragraph "TACI and BAFF-R").[10] Several studies have provided evidence of ***impaired BCR-mediated signaling*** in CVID patients, such as decreased upregulation of costimulatory molecules or defective calcium mobilization upon *in vitro* stimulation.[63-67] It was also shown that B cells of CVID patients, on average, have ***lower BAFF-R expression levels***.[68] Furthermore, in a subset of CVID patients, B cells showed a ***higher expression of the pro-apoptotic Fas receptor***, implicating increased apoptosis and further depletion of the peripheral B cell pool.[69,70] Note that defects in expression and/or signaling by the here-mentioned receptors were also reported in CVID patients with normal or increased B cell numbers, suggesting a broader functional impairment rather than a sole survival defect.[63-70] A reduction in total B cells, especially when very low, is associated with a more severe clinical course.[49,50,71] Importantly, (hypomorphic) mutations in *BTK* should be excluded in male patients with reduced B cell counts (see section 1.1.2.2).[72,73]

Patients with **increased total B cell numbers** have been less intensively studied. Possible mechanisms are increased bone marrow output, prolonged survival and/or higher peripheral B cell replication.[74] High total B cell counts have been associated with polyclonal lymphocytic infiltration and autoimmunity.[53]

1.5.2.2 *In vitro* B cell functionality

Besides changes in total B cell numbers, functional B cell alterations have also been documented in subgroups of CVID patients. For example, upon *in vitro* stimulation, some patients' B cells can produce normal amounts of IgG and IgM, whereas others can only secrete IgM or are unable to produce any immunoglobulin at all.[75] It is indeed puzzling that B cells of some CVID patients are able to produce normal immunoglobulin levels *in vitro* but not *in vivo*. One explanation could be that *in vivo* B cell activation by costimulatory cytokines and/or surface molecules is impaired in those patients.[76] Alternatively, suppression of

intracellular B cell signaling may be overcome by the supraphysiological concentrations of the *in vitro* stimuli.[76]

1.5.2.3 Peripheral B cell subsets

One of the most well described immunological abnormalities in CVID is an alteration in peripheral B cell subsets, mainly in class-switched memory B cells, marginal zone-like B cells, transitional B cells and CD21^{low} B cells (Figure 7) (see also section 1.1.2).

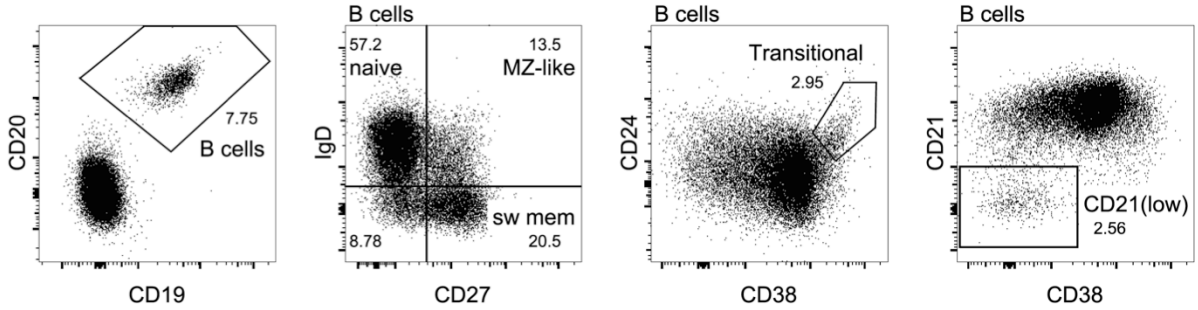


Figure 7. Peripheral B cell subsets. The first graph shows total B cells gated as CD19^{+}CD20^{+} cells in alive peripheral blood mononuclear cells (PBMCs). The second to fourth graphs depict B cell subsets gated on total B cells as indicated. Axes labels designate the markers used to discriminate ("gate") cell populations. Numbers on the graphs represent the frequency (percentage) of the parent population. Graphs are derived from an adult healthy subject. CD: cluster of differentiation, CD21(low): CD21^{low} B cells, Ig: immunoglobulin, MZ-like: marginal zone-like B cells, naive: naive mature B cells, sw mem: (class-)switched memory B cells, transitional: transitional B cells.

About 80% of patients have reduced levels of **class-switched memory (IgM^{-}IgD^{-}CD27^{+}) B cells** in the blood;[58-60,77] a feature first recognized in 2000 by Brouet *et al.*[78] In addition, several studies documented a depletion of **plasma cells in lymphoid organs** (bone marrow, lymph nodes, gut-associated lymphoid tissue) in the majority of the investigated patients.[61,79-84] Lack of switched memory B cells and plasma cells may indicate a block in late B cell development at the level of the GC reaction.[83] The assumption of a disturbed GC reaction in a subgroup of CVID patients is further supported by studies showing reduced rates of SHM in memory B cells.[77,85-88] However, not all patients with decreased switched memory B cells have impaired SHM.[87] Alternatively, the reduction in terminally differentiated B cells may be caused by increased apoptosis or disturbed migration upon stimulation during an immune response; a defect situated at the post-GC level.[89] Patients with low levels of switched memory B cells usually present a more severe clinical phenotype with a higher risk of developing noninfectious complications.[58-60]

Marginal zone-like (IgM^{+}IgD^{+}CD27^{+}) B cells, also known as natural effector B cells or non-switched memory B cells, are considered to be the circulating counterpart of splenic marginal zone B cells and can be generated in the absence of T cell help and a GC reaction.[90]

Marginal zone-like B cells are less consistently affected in CVID and are reduced in about 10% of patients.[91] In some study cohorts, reduced levels of marginal zone-like B cells were associated with respiratory tract infections and chronic lung disease.[87,91]

Subgroups of CVID patients show an expansion of functionally immature **transitional ($CD38^{high}CD24^{high}IgM^{high}$) B cells** and/or so-called **$CD21^{low}$ ($CD21^{low}CD38^{low}$) B cells** (Figure 7).[60] A relative increase in **transitional B cells** is believed to indicate a defect in peripheral B cell maturation (see section 1.1.2.2).[60] Elevated transitional B cells in CVID patients have been associated with the development of lymphadenopathy.[60] **$CD21^{low}$ B cells** are an unusual peripheral B cell subset and their ontogeny is unclear.[92-94] This subpopulation is thought to develop from memory B cells because their immunoglobulins have undergone CSR and SHM.[52,94] Furthermore, $CD21^{low}$ B cells express an altered chemokine receptor repertoire compared to other circulating B cell subsets, indicative of homing to inflammatory tissues instead of peripheral lymphoid tissues.[92] $CD21^{low}$ B cells show signs of previous activation and proliferation.[92,95] However, *in vitro* these cells have a poor proliferation capacity, which suggests a partial anergic status.[92,93] $CD21^{low}$ B cells are very infrequent in healthy individuals but increased levels have been reported in patients with autoimmune disorders (e.g. SLE, Sjögren syndrome, rheumatoid arthritis) and patients with chronic viral infections (e.g. HIV, hepatitis C virus).[93,94,96-98] In summary, $CD21^{low}$ B cells are regarded as chronically activated or exhausted B cells, derived from terminally differentiated B cells, that are expanded in conditions of chronic immune stimulation.[93,94,99,100] In CVID patients, increased $CD21^{low}$ B cells are associated with splenomegaly and autoimmune cytopenias.[60,101]

1.5.2.4 Classifications of CVID patients based on peripheral B cell subsets

Peripheral B cell immunophenotyping has been used to classify CVID patients into more homogeneous subgroups who share clinical phenotypes.[58-60] The main objective of these classifications was the identification of CVID patients at risk for certain noninfectious complications, in order to optimize treatment and follow-up.[29,46] Additionally, dividing CVID patients into subgroups could guide researchers to unravel the underlying pathophysiological mechanisms.[29,46]

The **Freiburg classification**, published in 2002, distinguished three groups of patients based on the levels of switched memory and $CD21^{low}$ B cells (Figure 8A).[58] The **Paris classification** from 2003 stratified CVID patients in three groups by means of total memory and switched memory B cell levels (Figure 8B).[59] The Paris groups showed poor agreement with the Freiburg groups, which may be due to methodological differences.[59]

In 2008, a multicenter European study (n=303) developed a consensus classification based on the two previous ones named **EUROclass**.[60] The EUROclass protocol distinguished more patient groups based on the levels of total B cells as well as switched memory, transitional and CD21^{low} B cell subsets (Figure 8C).[60] The associations found in the EUROclass trial corresponded partially with those found in the Freiburg and Paris studies. Severely reduced switched memory B cells were associated with splenomegaly and granulomatous disease, elevated CD21^{low} B cells with splenomegaly, and increased transitional B cells with lymphadenopathy.[60] For autoimmunity, no specific marker could be identified.[60]

The distribution of peripheral B cell subsets is dynamic and can sometimes show substantial changes over time. However, the clinical importance of this was not investigated.[102] Although the classifications based on peripheral B cell subsets have been valuable to some degree, in daily practice they turned out to be unsatisfactory. The associations between aberrant B cell subsets and clinical features were not consistently found in different CVID cohorts, and patients classified as being low risk were also seen to develop severe noninfectious complications.[42,103-105]

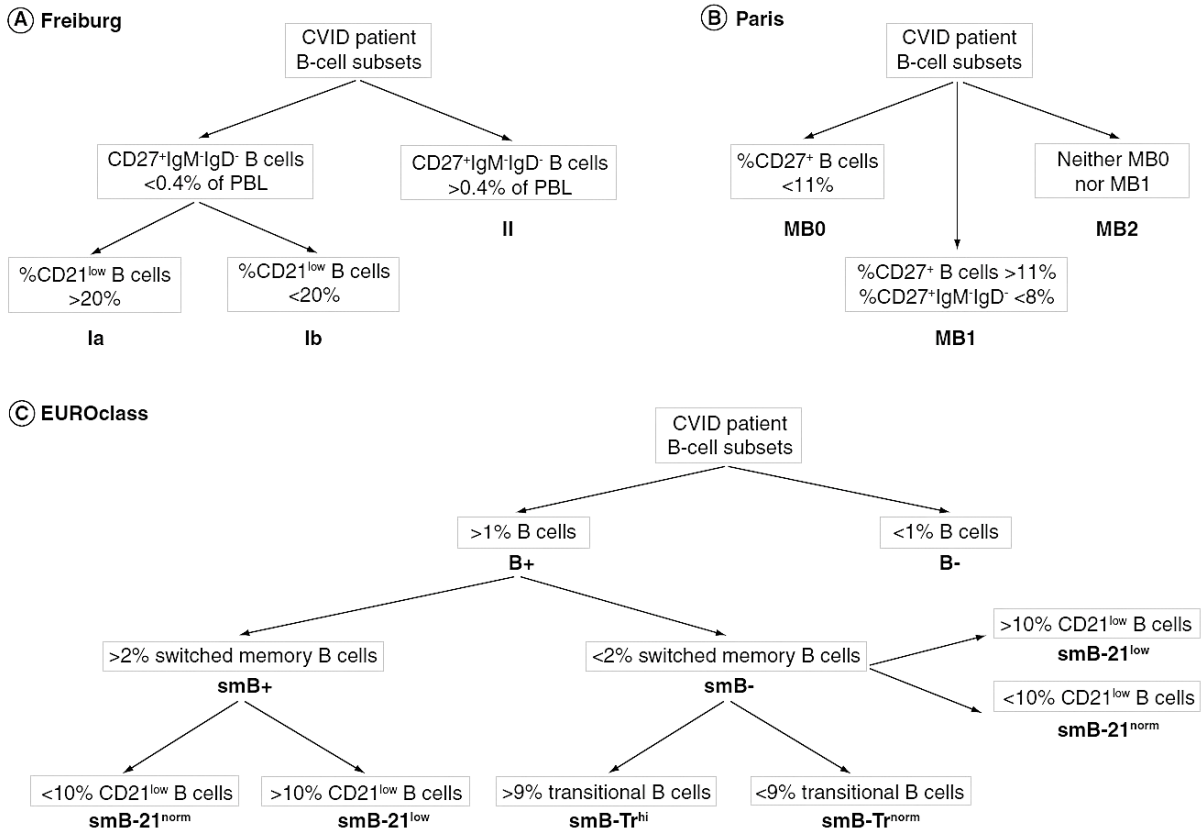


Figure 8. The Freiburg, Paris and EUROclass classifications. CVID patients are classified into clinically and immunologically more homogeneous groups based on flow cytometric phenotyping of peripheral B cell subsets. B cell subsets were expressed as percentages of peripheral blood lymphocytes (PBL) (Freiburg) or as percentages of total B cells (Paris, EUROclass). CD27$^+$ B cells: total memory B cells. CD27$^+$IgM$^-$IgD$^-$: switched memory B cells. *Adapted from Bergbreiter A and Salzer U. Expert Rev Clin Immunol. 2009;5(2):167-80.*

More recently, **Driessen *et al.*** distinguished five patient groups based on the composition, replication history and SHM rate of peripheral B cell subsets.[77] These five groups represent five main B cell patterns that each correspond with a block at a critical stage in B cell development (Figure 9): (1) B cell production, (2) early peripheral B cell maturation or survival, (3) B cell activation and proliferation, (4) germinal center, and (5) post-germinal center.[77] The work of Driessen *et al.* offered new pathophysiological insights on how defects at different stages in B cell development can lead to a final common pathway of CVID.[77] Moreover, the B cell pattern approach was also applicable to IPH patients, i.e. patients with hypogammaglobulinemia who do not fulfill all CVID diagnostic criteria (see section 1.3).[106] The B cell patterns showed little overlap with the EUROclass groups (Figure 8).[77] Noninfectious disease-related complications were mainly associated with defects in B cell production, early peripheral B cell maturation or B cell survival (patterns 1 and 2, Figure 9).[106]

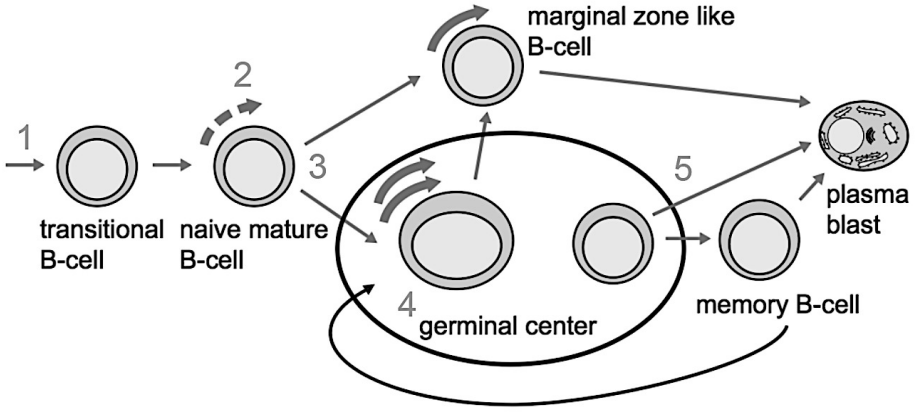


Figure 9. Peripheral B cell development with indication of the five stages of development block in CVID according to Driessen *et al.* The five B cell patterns delineate five immunological homogenous groups of CVID patients with different pathophysiological backgrounds. The curved arrows depict B cell proliferation. *Adapted from Driessen GJ et al. Blood. 2011;118(26):6814-23.*

1.5.2.5 B cell chemokine and Toll-like receptors

Migration (homing) of lymphocytes into and within lymphoid organs is regulated by chemokines and their receptors.[107] Important **chemokine receptors** expressed by B cells are CXCR4, CXCR5 and CCR7. CXCR4 is involved in homing to the bone marrow.[107] CXCR5 and CCR7 direct trafficking to the B cell follicles/zones and T cell zones, respectively, in peripheral lymphoid tissues (Figure 3).[107] CXCR4 expression on B cells was not significantly altered in CVID patients.[108] In contrast, expression of CXCR5 and CCR7 on naive and memory B cells was, on average, reduced in CVID patients.[45,108] These findings suggest a role for aberrant B cell migration in the pathogenesis of CVID.[108]

Toll-like receptors (TLRs) are membrane-spanning pattern recognition receptors that play a key role in the early innate immune response.[109] TLR7 and TLR9 are related endosomal receptors predominantly expressed by B cells and plasmacytoid dendritic cells (pDCs) (Figure 10) (see also section 1.5.4.2).[110] B cells differentially express these TLRs in the course of their life span: expression is very low in naive B cells, upregulated upon BCR-mediated activation, and constitutively high in memory B cells.[111] TLR7 and TLR9 signaling is triggered by specific microbial DNA and RNA motifs, and can induce T-independent B cell activation and antibody responses even in the absence of specific antigen (see also section 1.1.2.4).[110] Impaired TLR7 and TLR9 signaling in B cells and pDCs has been observed in a subgroup of CVID patients (Figure 10).[112-116] In those patients, TLR mRNA and protein expressions were normal and no genetic mutations or associated polymorphisms could be identified.[113,116,117] TLR7 and TLR9 signaling defects in CVID patients were independent of total B cell numbers or B cell memory phenotype.[112,113,115] However, patients with the lowest levels of switched memory B cells presented the most dysfunctional TLR-induced responses.[118,119] Taken together, dysfunction of TLR7 and TLR9 signaling appears to contribute to the pathophysiology of CVID, but is probably not the primary cause of disease.[119] Moreover, despite that TLRs are considered potent B cell activators, they do not seem to be essential for antibody production because patients with monogenic defects in TLR signaling molecules do not generally have antibody deficiencies.[118]

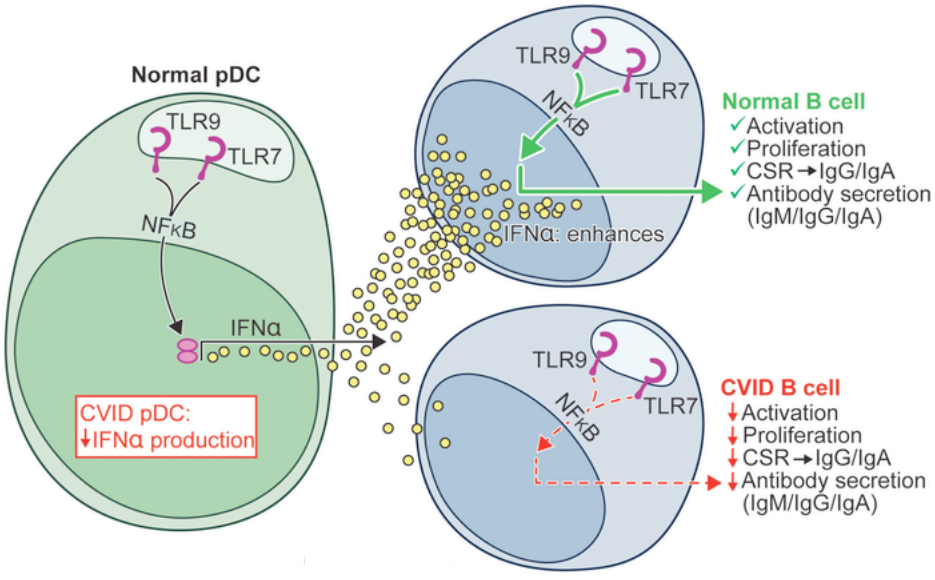


Figure 10. Toll-like receptor (TLR) 7 and TLR9 signaling in plasmacytoid dendritic cells (pDCs) and B cells are impaired in a subgroup of CVID patients. CSR: class switch recombination, CVID: common variable immunodeficiency, IFN: interferon, Ig: immunoglobulin, NFκB: nuclear factor of kappa light polypeptide gene enhancer in B cells. *Adapted from Mount Sinai Health System, 2015 (http://labs.icahn.mssm.edu/cunningham-rundles-lab/sample-page-2/common-variable-immune-deficiency/).*

1.5.3 T cell abnormalities

In parallel with the identification of B cell abnormalities, numerous studies have provided evidence that the T cell compartment is also misbalanced in CVID. Note that most of the T cell data were generated before the introduction of the revised ESID registry criteria for CVID in 2014, which for the first time emphasized the exclusion of patients with more severe T cell deficiency (see section 1.3). It would be of interest to reassess the T cell findings in a better delineated patient population, as we did in chapter 3. Still, much can be learned from the available reports on T cell abnormalities in CVID patients.[120]

1.5.3.1 Peripheral CD4$^+$ T cells

CD4$^+$ T lymphopenia is one of the most frequently reported T cell abnormalities in CVID and is estimated to affect up to one third of patients. Reduced CD4$^+$ T cell numbers in CVID patients are associated with a higher risk of disease-related complications, especially granulomatous disease and splenomegaly.[121-124] The decrease in CD4$^+$ T cells can extend to all subsets but is predominately restricted to the **naive pool** (Figure 11).[54,122-124]

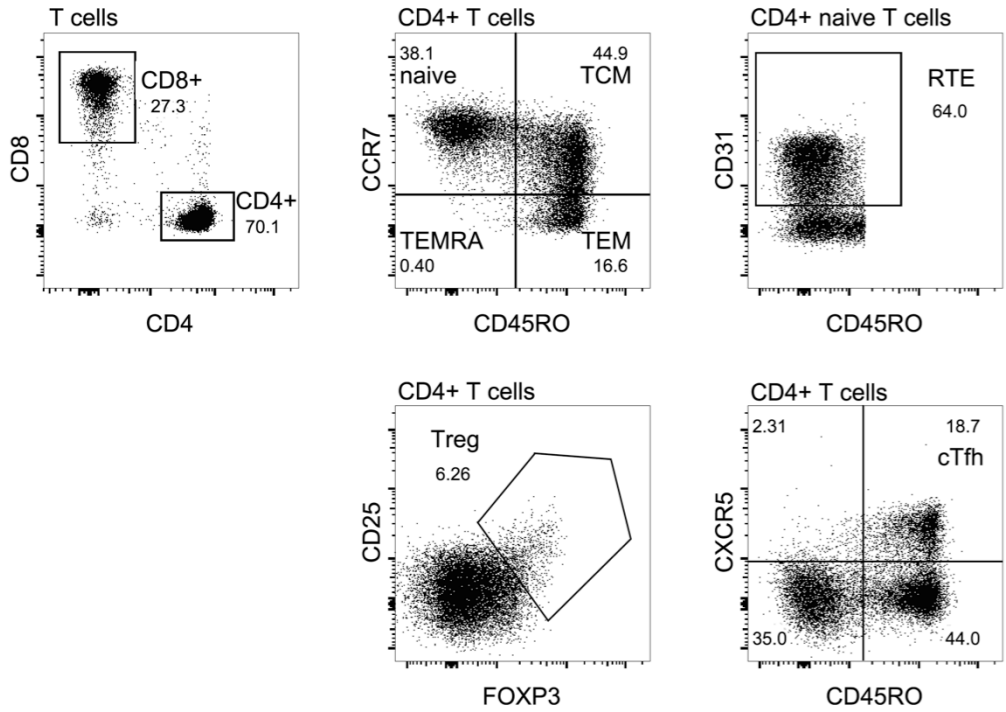


Figure 11. Peripheral CD4$^+$ T cell subsets. The first graph shows CD8$^+$ and CD4$^+$ T cells gated in the total T cell population. The remaining graphs depict CD4$^+$ T cell subsets as indicated. Axes labels designate the markers used to discriminate ("gate") cell populations. The parent population is depicted on top of each graph. Numbers represent the frequency (percentage) of the parent population. Graphs are derived from an adult healthy subject. CD: cluster of differentiation, cTfh: circulating follicular helper T cells, naive: naive T cells, RTE: recent thymic emigrants, TCM: central memory T cells, TEM: effector memory T cells, TEMRA: effector memory RA T cells, Treg: regulatory T cells.

Giovannetti *et al.* demonstrated that the degree of naive $CD4^+$ T cell loss was linked to the degree of overall T cell dysfunction and disease severity.[122] Based on these findings, the authors proposed an alternative CVID classification scheme using naive $CD4^+$ T cell percentages as single parameter (Figure 12).[122] A more severe depletion in naive $CD4^+$ T cells was associated with more severe T and B cell abnormalities as well as a more severe clinical picture, especially splenomegaly.[122]

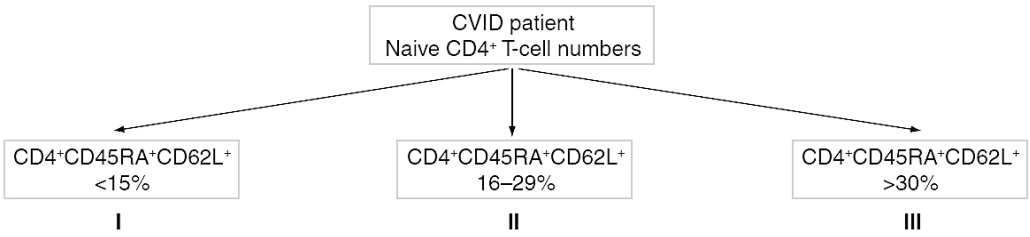


Figure 12. The naive $CD4^+$ T cell classification by Giovannetti *et al.* CVID patients are classified into clinically and immunologically more homogeneous groups based on the proportion of peripheral naive $CD4^+$ T cells ($CD4^+CD45RA^+CD62L^+$). *Adapted from Bergbreiter A and Salzer U. Expert Rev Clin Immunol. 2009;5(2):167-80.*

Despite the fact that decreased naive $CD4^+$ T cells were correlated with decreased switched memory B cells and increased $CD21^{low}$ B cells (see section 1.5.2.3), the Giovannetti groups showed little concordance with the previously published Freiburg groups (Figure 8A).[122] Similar to the classifications based on peripheral B cell subsets, the naive $CD4^+$ T cell classification failed to capture the entire CVID picture and did not allow a satisfactory prediction of the risk of disease-related complications.[46]

Peripheral $CD4^+$ T cells are mainly replenished by output from the thymus, whereas $CD8^+$ T cell numbers are predominantly conserved by proliferation of the existing peripheral pool.[122] Hence, a decrease of naive $CD4^+$ T cells in CVID patients is suggestive of **reduced thymic output**. Additional evidence supporting thymic dysfunction in CVID patients includes a reduction of peripheral T cells carrying a T cell receptor excision circle (TREC),[74,125,126] reduced levels of $CD4^+$ recent thymic emigrant ($CD45RA^+CD31^+$) cells (Figure 11),[62,122,124] and the presence of a more restricted T cell receptor (TCR) repertoire.[122,127] Moreover, several studies provided evidence of **increased T cell apoptosis**, which could contribute to the (naive) $CD4^+$ T lymphopenia.[122,128,129]

A decrease of peripheral blood **regulatory T (Treg) cells ($CD4^+CD25^+FOXP3^+$** or **$CD4^+CD25^+CD127^-$)** has also been described in a proportion of CVID patients (Figure 11).[130-135] Treg cells are known for their modulating role in immune responses, crucial for preventing autoimmune disease and chronic immune activation.[136] It is, therefore, not surprising that reduced Treg cells in CVID patients are associated with disease-related complications such

as autoimmunity, granulomatous disease, lymphadenopathy and splenomegaly.[130-135] Low Treg cells are also correlated with reduced switched memory B cells and increased $CD21^{low}$ B cells, two other known risk factors for disease-related complications (see section 1.5.2.3).[132,133] Treg cells either directly originate from the thymus or are peripherally induced from $CD4^+$ T cells.[136] Hence, decreased Treg cell levels in CVID patients could fit the broader picture of a disturbed thymopoiesis.[120] Alongside a quantitative shortage, Treg cells isolated from CVID patients with autoimmunity also demonstrated a functional deficit. In particular, they expressed lower levels of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) that is essential for their regulatory function (see also section 1.6.2.1, paragraph "CTLA-4 deficiency"), and showed an attenuated suppressive activity when co-cultured with autologous $CD4^+CD25^-$ T cells.[134,137,138]

Conflicting literature exists regarding the role of **follicular helper T (Tfh) cells** in the pathogenesis of CVID. As explained in section 1.1.2.3, Tfh cells orchestrate the GC reaction where B cells undergo terminal differentiation into high-affinity antibody-secreting plasma cells and memory B cells.[8] Patients with monogenic inborn defects in inducible T cell co-stimulator (ICOS) have reduced Tfh cells and abrogated GC reactions (see section 1.6.2.1, paragraph "ICOS deficiency").[139] In contrast, others and we have found increased levels of **circulating Tfh ($CD4^+CD45RO^+CXCR5^+$) cells** in a subgroup of CVID patients in whom a genetic diagnosis had not been determined (Figure 11) (see Chapter 3).[45,124,140] Furthermore and analogous to B cells, T cell expression of the chemokine receptor CCR7 was found to be reduced in CVID patients (see section 1.5.2.5).[108] The latter suggests impaired migration of T cells to peripheral lymphoid tissues, which could contribute to the increased levels of circulating Tfh cells in some CVID patients. However, possible disease-influencing mechanisms of Tfh cells in CVID await further study.

1.5.3.2 Peripheral $CD8^+$ T cells

As mentioned above, the conservation of peripheral $CD8^+$ T cell numbers is less dependent on thymus activity than its $CD4^+$ counterpart.[122] Although $CD8^+$ T cells can occasionally be reduced,[141,142] up to 30% of CVID patients have an **increase in $CD8^+$ T cells**.[120,142-147] The latter was found to be correlated with a reduction in Treg cells and switched memory B cells and a higher risk of developing disease-related complications.[55,124,135,143] The increased $CD8^+$ T cells in CVID patients exhibit high levels of activation markers (e.g. HLA-DR, CD69), and mainly include **effector memory ($CD45RO^+CCR7^-$)** and **terminally differentiated senescent ($CD45RO^-CCR7^-$)** subsets (Figure 13).[120,135,143,148-151] This immunophenotypical

profile of $CD8^+$ T cells is indicative of chronic activation and suggests **T cell exhaustion**.[149,150]

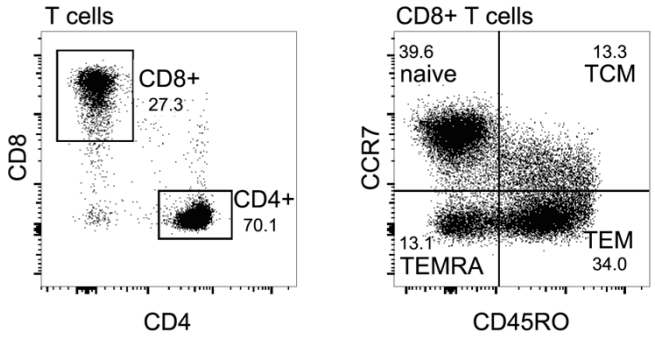


Figure 13. Peripheral $CD8^+$ T cell subsets. The first graph shows $CD8^+$ and $CD4^+$ T cells gated in the total T cell population. The second graph depicts $CD8^+$ T cell subsets as indicated. Axes labels designate the markers used to discriminate ("gate") cell populations. Numbers on the graphs represent the frequency (percentage) of the parent population. Graphs are derived from an adult healthy subject. CD: cluster of differentiation, naive: naive T cells, TCM: central memory T cells, TEM: effector memory T cells, TEMRA: effector memory RA T cells.

To date, it is unclear why $CD8^+$ T cells in a significant proportion of CVID patients are expanded and driven towards exhaustion. Marashi *et al.* proposed a role for cytomegalovirus (CMV) in chronic activation of $CD8^+$ T cells and subsequent development of inflammatory complications.[152,153] In their cohort (n=71), CVID patients with unexplained enteropathy and/or lymphoproliferation had higher levels of CMV-specific $CD8^+$ T cells compared to healthy controls, while Epstein-Barr virus (EBV)-specific $CD8^+$ T cells were not elevated.[152] Furthermore, CMV-specific $CD8^+$ T cells showed greatly increased activation and proliferation markers, suggesting a persistent and unregulated $CD8^+$ T cell response.[152,153] However, direct causality between CMV, $CD8^+$ T cell expansion and inflammatory complications could not be demonstrated and bystander activation of CMV-specific $CD8^+$ T cells could not be excluded.[152,153] Besides an underlying infectious driver, T cell exhaustion in CVID could also be the result of an exaggerated T cell response in a non-infectious context like autoimmunity.[120] A possible role for autoimmunity in chronic $CD8^+$ T cell activation and expansion in CVID patients remains to be investigated.[120]

1.5.3.3 T cell proliferation and cytokine production

In 40% to 70% of CVID patients, **T cell proliferation** responses to antigens (e.g. tetanus toxoid) or TCR antibodies are partially decreased.[43,49,154,155] In some cohorts, suboptimal T cell proliferation responses were more pronounced in CVID patients with disease-related complications.[43] The cause of impaired T cell proliferation is usually not known, but could be related to a state of T cell exhaustion in a subset of cases.[120,156]

T cell cytokines play an important role in B cell differentiation and antibody production (also see section 1.1.2.3). A plethora of studies have attempted to identify distinct cytokine profiles in CVID patients. However, conflicting data has been reported over the years, which was recently put together by Varzaneh *et al.* in a comprehensive review.[157] In brief, there are indications of a cytokine imbalance in at least a portion of CVID patients, supported by the aberrant production of interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-21, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and other cytokines upon *in vitro* T cell stimulation.[157] Depending on the study, the same cytokines could be either decreased or increased (e.g. IL-4, IL-10, IFN-γ), while other cytokines showed more consistency regarding the direction in which they deviated (e.g. decreased IL-2).[157] This divergent cytokine image could be partially due to the heterogeneity of the CVID population and partially to the methodological differences of the studies (e.g. type of stimuli, cutoff levels).[157] In addition, some authors reported that cytokine production might be influenced by polymorphisms in the coding and promoter regions of cytokine-coding genes.[158-160] Immunoglobulin replacement therapy has been shown to affect serum cytokine concentrations to some degree,[161,162] but it is uncertain whether this would also influence *in vitro* cytokine production.

Interestingly, Wong *et al.* postulated that the cytokine signature in some CVID patients resembled an early phase of T cell exhaustion.[120] In particular, the combination of a decreased IL-2 and an increased TNF-α and IFN-γ production reminded the authors of the cytokine alterations described in patients with T cell exhaustion caused by chronic viral infections.[120] **Reduced IL-2 production** has been a recurrent finding in CVID patients for over 30 years.[157] Preliminary studies from the 1990s demonstrated that treatment with recombinant IL-2 might be beneficial in selected CVID patients.[163-166] In those studies, treated patients showed enhanced T cell function, increased antibody responses, and a reduction of severe infections.[163-166] Trials in larger cohorts were not performed. In conclusion, future studies are necessary to dissect the conflicting cytokine data and to identify patient groups that might benefit from cytokine-targeted therapies.[157]

1.5.4 Innate immune cell abnormalities

A limited number of studies have described alterations in cells of the innate immune system in subgroups of CVID patients. This section gives an overview of current knowledge on innate immune cell dysfunction in CVID.

1.5.4.1 Monocytes

Blood monocytes play important roles in the initiation and resolution of inflammation during the innate immune response (Figure 14).[167] They mainly function through phagocytosis,

generation of reactive oxygen species (ROS), secretion of inflammatory cytokines, and activation of cells of the adaptive immune system.[167]

Arguments for **chronic hyperactivity of blood monocytes** have been found in a selection of CVID patients.[168-170] In earlier studies, isolated monocytes from some CVID patients showed an enhanced ROS generation *in vitro*, which was associated with increased serum levels of TNF-α and soluble TNF receptors in those patients.[168,169] In addition, raised serum levels of neopterin, a monocyte/macrophage activation marker, were observed in several patient cohorts.[130,168,170] Monocyte hyperactivity in CVID patients was often associated with a generalized immune dysregulation state characterized by reduced switched memory B cells, increased $CD21^{low}$ B cells, expansion of activated effector T cells, and/or low $CD4^+$ T cells.[130,168,171]

Furthermore, some CVID patients demonstrated an **altered monocyte cytokine profile with higher levels of IL-12**.[172,173] This cytokine environment does not favor antibody production and may thus contribute to the disease process of CVID.[172,173]

Finally, **impaired phagocytosis by monocytes** in a subset of CVID patients may hamper both elimination of pathogens and antigen presentation to lymphocytes.[174]

Altogether, monocyte dysfunction could be involved in the pathogenesis of CVID in a subgroup of patients. In most cases, impaired monocyte function seems to be part of an overall dysregulation or hyperactivity of the immune system, although a monocyte-intrinsic defect in a limited number of patients cannot be excluded.

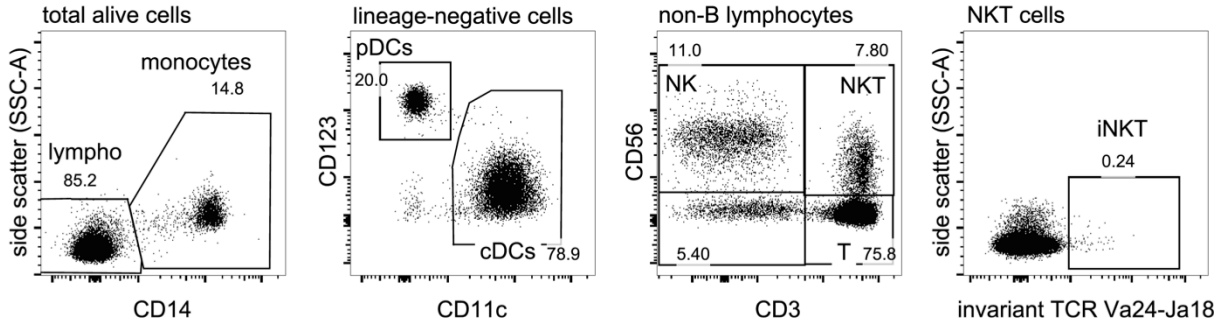


Figure 14. Peripheral innate immune cell subsets. Graphs depict innate immune cell subsets gated on peripheral blood mononuclear cells (PBMCs) as indicated. Axes labels represent the markers used to discriminate ("gate") cell populations. The parent population is shown on top of each graph. Numbers represent the frequency (percentage) of the parent population. Graphs are derived from an adult healthy subject. CD: cluster of differentiation, cDCs: conventional dendritic cells, iNKT: invariant natural killer T cells, lympho: lymphocytes, NK: natural killer cells, NKT: natural killer T cells, pDCs: plasmacytoid dendritic cells, T: T cells, TCR: T cell receptor.

1.5.4.2 Dendritic cells

Dendritic cells (DCs) occupy a central position in the immune system as they form a bridge between innate and adaptive responses.[175] They are subdivided in three main groups: **conventional DCs (cDCs)**, **plasmacytoid DCs** (**pDCs**; previously mentioned in section 1.5.2.5), and **monocyte-derived DCs (moDCs)** (Figure 14).[175] **cDCs** are specialized in antigen presentation and priming of naive T cells, and are, therefore, crucial for initiating T-dependent antibody responses (see section 1.1.2.3).[175] Note that cDCs can also directly stimulate B cells during T-independent antibody responses through the secretion of BAFF and APRIL (see section 1.1.2.4). **pDCs** are mainly involved in antiviral immunity by secreting abundant type I IFNs.[175] IFN-α, a type I IFN, can also enhance antibody responses in the absence of T cell help.[176] **moDCs** are generated *in vitro* from monocytes cultured with granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4.[175]

Peripheral blood cDC and pDC numbers were found to be **decreased** in a proportion of CVID patients. This was most pronounced in patients with decreased switched memory B cells and disease-related complications such as granulomatous disease, autoimmunity and splenomegaly.[177-181]

As mentioned above (see section 1.5.2.5), **TLR7 and TLR9 signaling in pDCs** (and B cells) is **impaired** in subgroups of CVID patients.[112-116]

In some cases, DCs derived from patients' monocytes (**patient moDCs**) showed severely **disrupted differentiation and maturation** with poor upregulation of costimulatory molecules (CD80, CD86, HLA-DR), decreased IL-12 secretion and impaired T cell activation in co-cultures.[182-184] The patient moDC results should, however, be interpreted with caution since the authenticity of moDCs is controversial and they may not be a proper correlate of naturally occurring DCs.[175] To determine whether myeloid-type DCs are truly malfunctioning, it would be of interest to revisit the moDC data in *bona fide* cDCs of CVID patients.

1.5.4.3 Natural killer cells

Natural killer (NK) cells are innate lymphocytes with cytotoxic capacities analogous to those of $CD8^+$ cytotoxic T cells (Figure 14).[185] NK cells express a variety of activating and inhibitory surface receptors.[185] Some of these receptors sense alterations in MHC class I expression on host cells, which may be induced by intracellular pathogens. Others recognize (less well defined) changes in cell-surface glycoprotein composition induced by metabolic stresses such as infection or malignant transformation. Hence, NK cells are, similar to $CD8^+$ T cells, important for the destruction of (virus-) infected and malignantly transformed cells.[185]

Absolute numbers of circulating NK cells were markedly **reduced** in a subgroup of CVID patients.[181,186,187] Curiously, patients with low NK cell counts did not appear to have an

increased susceptibility to viral infections.[181,186,187] Moreover, *in vitro* NK cell cytotoxicity analyses were normal, suggesting that the function of the remaining NK cells could compensate for the low numbers.[186,187] Despite the lack of obvious clinical consequences, NK cell deficiency in CVID patients may further compromise an already malfunctioning immune system by undermining control of viral infections and tumor immunosurveillance.[188]

1.5.4.4 Natural killer T cells

Natural killer T (NKT) cells are lymphoid cells that express NK cell receptors as well as CD3 (i.e. T cell co-receptor) and a TCR, and are therefore situated at the interface between adaptive and innate immunity (Figure 14).[189] They possess an exceptional TCR of limited diversity that recognizes (glyco)lipids presented by the MHC-like molecule CD1d.[189] NKT cells can produce a wide array of cytokines and chemokines, and appear to have an immunoregulatory function.[189] Moreover, NKT cells can enhance T-dependent antibody responses through interaction with CD1d on B cells.[190]

Total NKT cells have only been examined in a limited number of CVID patients.[187,191] In a first cohort (n=17), CVID patients had, on average, **lower numbers of circulating NKT cells** compared to healthy controls.[191] NKT cells in those patients were skewed towards an activated phenotype, but associations with clinical features were not reported.[191] In a second CVID patient cohort (n=20), no differences in total NKT cells were found, regardless of disease severity.[187]

Invariant NKT (iNKT) cells are a subset of NKT cells that express an invariant TCR α chain with one of a limited number of β chains, which recognize lipid antigens in the context of CD1d (Figure 14).[189] Recent studies have described a significant **reduction in peripheral blood iNKT cell numbers**, concerning up to 75% of investigated CVID patients.[181,192-194] Reduced iNKT cells in those patients was associated with impaired iNKT cell function, decreased switched memory B cells and lymphoproliferation.[192,193]

It is unclear how a decrease in blood (i)NKT cells fits into the pathophysiology of CVID, whether it concerns primary, cell-intrinsic, defects or secondary phenomena due to, for example, sequestration in inflamed tissues.

1.5.4.5 Innate lymphoid cells

Innate lymphoid cells (ILCs) are a recently identified group of innate immune cells belonging to the lymphoid lineage.[195] In contrast to lymphocytes of the adaptive immune system, ILCs do not have a recombined antigen receptor and are activated in the early phases of an immune response.[196] ILCs are present in various tissues, but seem to be particularly important for immune defense at mucosal barriers.[196] ILCs are divided in three groups based

on their cytokine and transcription factor profiles, which are analogous to the profiles of their adaptive Th1, Th2 and Th17 counterparts, respectively.[196] The above-described cytotoxic NK cells (section 1.5.4.3), although originally considered a separate cell population, are now categorized under group 1 ILCs.[196]

Thus far, a possible role of ILCs in CVID has only been investigated by a single study (n=55).[197] CVID patients with inflammatory and/or autoimmune complications had an **expanded population of IFN-γ^+ IL-17A$^+$ IL-22$^+$ ILCs** both in the peripheral blood and in biopsies of mucosal lymphoid tissues.[197] These findings suggested that ILCs might be involved in inflammatory reactions and mucosal damage in at least a subgroup of CVID patients.[197] Future study is required to confirm these data and to further define the place of ILCs in CVID pathogenesis.[197]

1.5.5 Overview and concluding remarks

A summary of the most important immunological abnormalities described in the CVID population is given in Table 2.

Table 2. Summary of the most important immunological abnormalities reported in CVID.

B cells	T cells	Innate immune cells
↓ total B cells	↓ naive (or total) CD4$^+$ T cells	Monocytes :
↓ B cell bone marrow development	↓ thymic output :	↑ activity
↓ BCR signaling	↓ TRECs	↑ IL-12 secretion
↓ BAFF-R expression (survival)	↓ CD4$^+$ recent thymic emigrants (CD45RA$^+$CD31$^+$)	↓ phagocytosis
↑ Fas expression (apoptosis)	↑ apoptosis	↓ conventional DCs
↓ class-switched memory B cells (IgM$^-$IgD$^-$CD27$^+$)	↓ TCR repertoire	↓ plasmacytoid DCs
↓ plasma cells in lymphoid organs	↓ Treg cells (CD4$^+$CD25$^+$FOXP3$^+$ / CD127$^-$)	↓ TLR7 and TLR9 signaling in pDCs
↓ somatic hypermutation	↓ Treg suppressive activity	↓ maturation monocyte-derived DCs
↓ marginal zone-like B cells (IgM$^+$IgD$^+$CD27$^+$)	↓ or ↑ circulating Tfh cells (CD4$^+$CD45RO$^+$CXCR5$^+$)	↓ NK cells
↑ transitional B cells (CD38^{high}CD24^{high}IgM^{high})	↑ CD8$^+$ T cells (mainly effector subsets)	↓ NKT cells
↑ CD21^{low} B cells (CD21^{low}CD38^{low})	↓ T cell proliferation responses to antigens and TCR antibodies	↓ invariant NKT cells
↓ CXCR5 and CCR7 expression	cytokine imbalance (e.g. ↓ IL-2)	↑ IFN-γ^+ IL-17A$^+$ IL-22$^+$ ILCs
↓ TLR7 and TLR9 signaling		

↓ : decreased, ↑ : increased, BAFF-R: B cell activating factor belonging to the TNF family - receptor, BCR: B cell receptor, CD: cluster of differentiation, CVID: common variable immunodeficiency, DCs: dendritic cells, IFN: interferon, Ig: immunoglobulin, IL: interleukin, ILCs: innate lymphoid cells, NK: natural killer, NKT: natural killer T, pDCs: plasmacytoid dendritic cells, TCR: T cell receptor, Tfh: follicular helper T, TLR: Toll-like receptor, TRECs: T cell receptor excision circles, Treg: regulatory T.

Note that each of these laboratory features is only present in a subgroup of patients, and that the parameters may also be normal or even deviant in the opposite direction in other CVID

patients. Remarkably, immunological abnormalities across all parts of the immune system seem to cluster together in patients suffering from severe disease-related complications.[46] It is, therefore, suspected that at least a fraction of these abnormalities are secondary to a condition of immune dysregulation and chronic inflammation.[46] Long-term prospective studies with regular follow-up of immunological parameters might help discriminate primary from secondary phenomena in CVID patients.[36]

1.6 Genetic basis of CVID

Adapted from: Bogaert DJ, Dullaers M, Lambrecht BN, Vermaelen KY, De Baere E, Haerynck F. Genes associated with common variable immunodeficiency: one diagnosis to rule them all? J Med Gen. 2016;53(9):575-90.

1.6.1 "Out of one, many"

Based on the fact that CVID is a diagnosis of exclusion together with the broad spectrum of clinical manifestations and immunological abnormalities seen in CVID patients, it quickly became apparent that this diagnosis does not cover one or a few disorders but many. This perception has been confirmed by the gradual elucidation of the genetic basis of CVID.

The majority of CVID cases occur sporadically.[29] About 5-25% of patients have a positive family history, of which most demonstrate an autosomal dominant inheritance.[29] In contrast to other PIDs, a **monogenic cause** has only been identified in **2-10% of patients** diagnosed with CVID (Figure 15).[29,198] Thus far, 24 genes have been implicated in monogenic forms of CVID (Table 3). Each of these genetic subtypes is rare to very rare (Figure 15). The first CVID disease genes were discovered with a candidate gene approach because of their central role in B cell biology evidenced by single-gene knockout mice.[199-202] This approach generally uncovered autosomal recessive genes. In recent years, NGS technologies have accelerated the identification of both autosomal recessive and dominant CVID disease genes (see also section 1.2.1.1). In parallel, it has become clear that the clinical diagnosis of CVID is an umbrella covering several genetic subtypes. In fact, many genes initially reported as CVID disease genes are now considered to be responsible for distinct disease entities (Table 3). Moreover, there is accumulating evidence that at least a subgroup of CVID patients have a complex (multifactorial) rather than a monogenic etiology (see also section 1.2.1.4).[29,198,203]

Current knowledge on the genetic basis of CVID is discussed in the next sections, covering both monogenic and complex forms, and linking with clinical and immunological phenotypes.

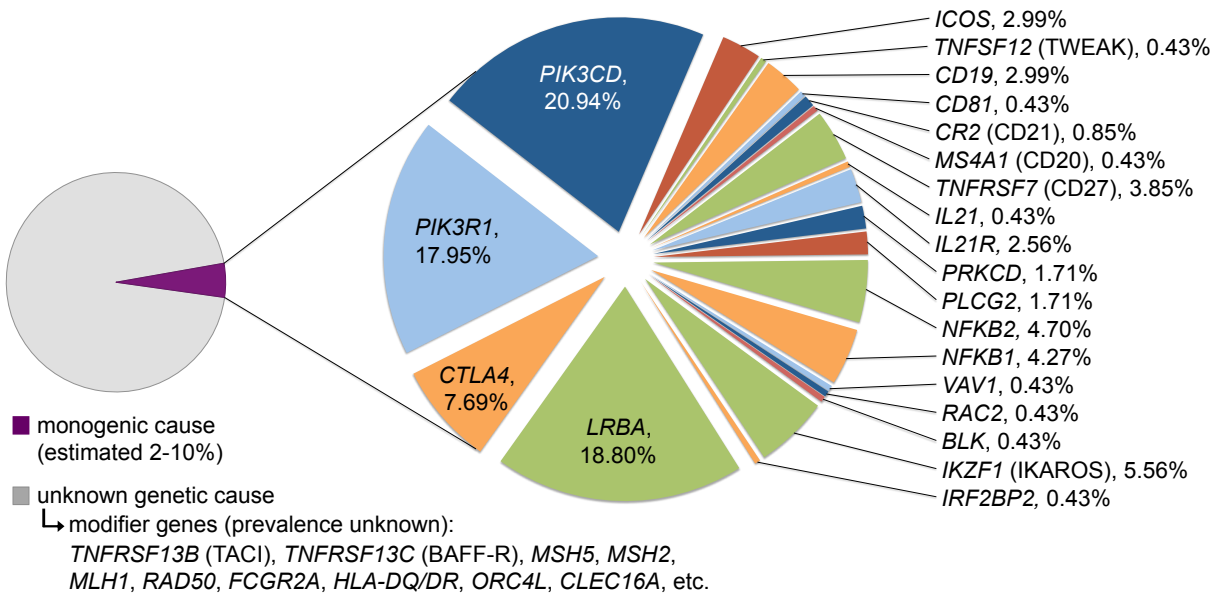


Figure 15. Estimated proportion of each disease gene within the CVID population based on published cases.

1.6.2 Genes associated with monogenic forms of CVID

Table 3, at the end of this section, gives an overview of the genes implicated in monogenic CVID. Most genes are associated with variable (clinical and immunological) expressivity and some genes also with incomplete penetrance. As mentioned in section 1.2.1.4, the molecular basis of variable expressivity and incomplete penetrance is poorly understood and may involve modifier genes, epigenetic changes and/or environmental factors.[15,26]

1.6.2.1 Genes encoding receptors and ligands

ICOS deficiency

ICOS is a T cell surface receptor that belongs to the CD28/CTLA-4 family (Figure 16).[199] Reciprocal ICOS-ICOS ligand (ICOS-L) interactions are essential for GC formation and terminal B cell differentiation (see section 1.1.2.3), effector T cell responses, and immune tolerance.[199] *ICOS* was the first disease gene identified for monogenic forms of CVID, using a candidate gene approach based on prior evidence from single-gene knockout mice.[199] Hitherto, biallelic *ICOS* mutations resulting in complete loss of protein expression have been reported in seven families.[199,204-207] Haplotype analysis in the four German/Austrian families segregating an identical *ICOS* mutation was indicative for a common founder.[204,208]

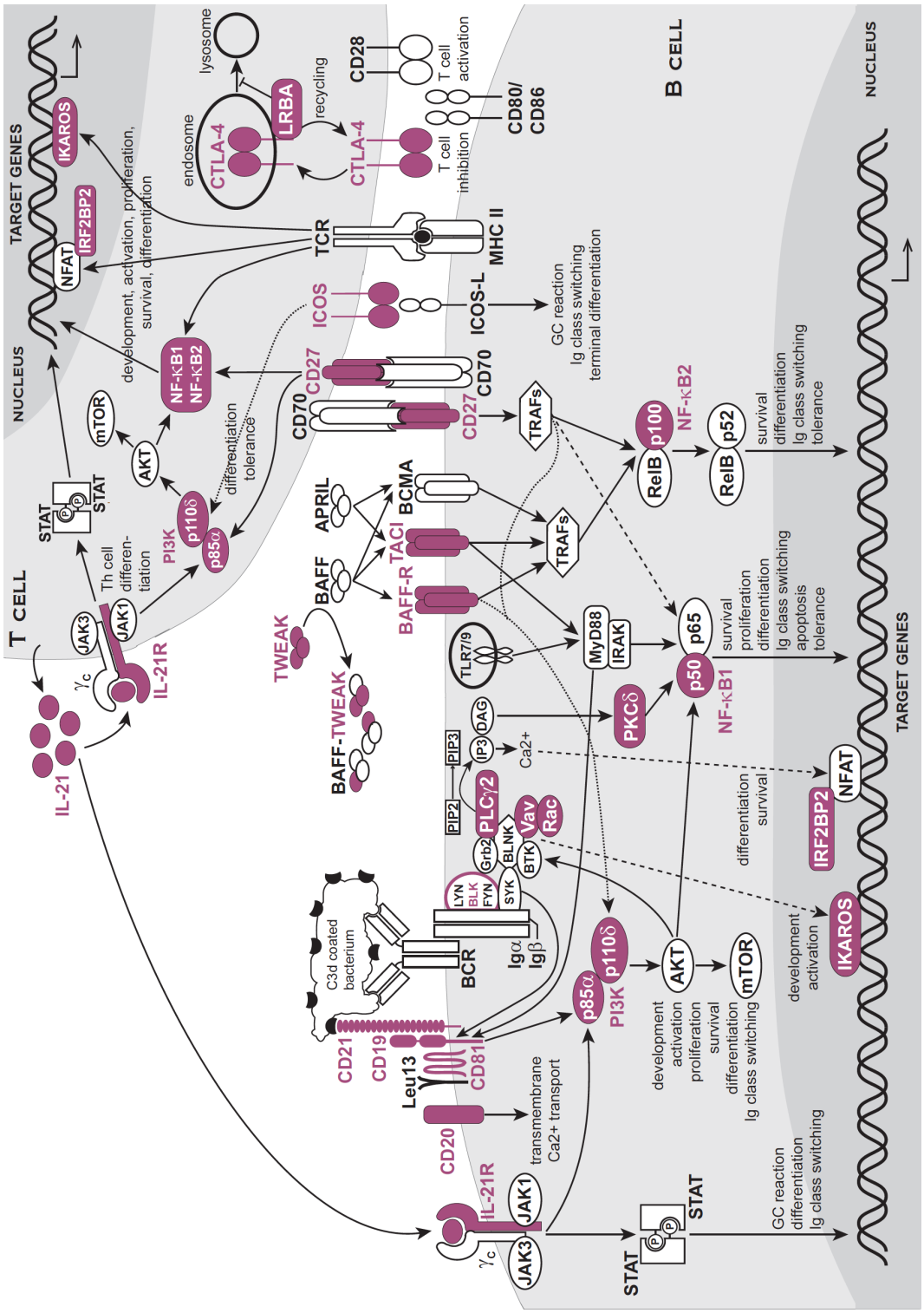


Figure 16. Scheme comprising proteins encoded by CVID-associated genes (purple). Only the most important interacting molecules, pathways and functions relevant to this introduction are depicted. See text for details.

ICOS-deficient patients had a variable phenotype with variable age of onset and severity (Table 3).[199,204-207] They commonly presented recurrent respiratory tract infections and autoimmune complications.[199,204-207] Patients with two novel *ICOS* mutations published in 2015 extended the clinical spectrum: early-onset inflammatory bowel disease, hepatomegaly with raised liver enzymes, CMV viremia, and *Pneumocystis jirovecii* pneumonia.[206,207] Enteropathy in one ICOS-deficient patient resolved after hematopoietic stem cell transplantation (HSCT) while diarrhea persisted in his non-transplanted sister. This suggests that inflammatory gut complications are disease-intrinsic.[206]

All ICOS-deficient patients had very low to absent memory B cells and some also showed a loss of bone marrow plasma cells.[199,204-208] This might be due to defective GC reactions in the absence of ICOS signaling.[208] ICOS-deficient patients also demonstrated varying degrees of T cell defects (Table 3). In contrast to the first-reported German/Austrian families, the Japanese, Kuwaiti, and Pakistani sibling pairs demonstrated pronounced T cell defects with viral and opportunistic infections resembling CID rather than CVID.[199,204-208] Therefore, *ICOS* mutations are no longer considered to cause a pure CVID phenotype but result in a separate disease entity (ICOS deficiency).[206,207]

TACI and BAFF-R

TACI (encoded by *TNFRSF13B*), BAFF-R (encoded by *TNFRSF13C*) and B cell maturation antigen (BCMA) are members of the TNF receptor superfamily (TNFRSF) important in peripheral B cell homeostasis.[10] These receptors engage two ligands: BAFF and APRIL (Figure 16). Both ligand and receptor oligomerization are necessary for optimal downstream signaling.[10] The TACI/BAFF-R/BCMA/BAFF/APRIL system signals through many pathways,[10,209] of which a selection is depicted in Figure 16. How the TACI/BAFF-R/BCMA/BAFF/APRIL system fine-tunes B cell homeostasis and the degree of mutual redundancy remain incompletely understood.[10,209] TACI mediates CSR to IgA and IgG, differentiation and survival of plasma cells, and T-independent responses to polysaccharide antigens (see also section 1.1.2.4). TACI also acts as an immunoregulator involved in central B cell tolerance and inhibiting peripheral B cell expansion.[10,209] BAFF-BAFF-R signaling promotes peripheral B cell survival and maturation in synergy with BCR signaling (see also section 1.5.2.1).[10] BCMA plays a role in long-term plasma cell survival in bone marrow.[10]

Variants in the genes encoding TACI and BAFF-R have been identified in CVID patients by means of a candidate gene approach based on single-gene knockout mice.[200-202] Although initially thought to be fully penetrant, it is currently believed that monoallelic *TNFRSF13B* and mono- and biallelic *TNFRSF13C* variants are on itself not sufficient to cause a CVID phenotype (modifier genes, see section 1.2.1.4).[210-214]

TNFRSF13B (encoding TACI) variants

Biallelic and monoallelic loss-of-function variants in *TNFRSF13B* have been registered in at least 2147 patients based on the Jeffrey Modell Centers Global Network report.[13] Biallelic *TNFRSF13B* variants have always been associated with some degree of antibody deficiency,[103,200,201,211,212,215-218] except for a homozygous C104R variant in a 25-year-old member of a CVID-affected family who was asymptomatic and had normal Ig levels at time of the study.[216] The latter individual could still have developed antibody deficiency later in life, however.[216] In contrast, monoallelic *TNFRSF13B* variants have also been detected in asymptomatic relatives and in 1-2% of the general population.[210-213,216,218]

A large variety of variants, mostly missense and nonsense variants, located in all domains of the TACI protein have been reported.[103,200,201,210-213,215-220] The monoallelic missense variants C104R and A181E account for 80% of all *TNFRSF13B* variants in CVID patients.[103,200,201,210-213,215-220] The majority of *TNFRSF13B* variants do not or only slightly reduce TACI protein expression.[220] In particular, C104R interferes with ligand binding and A181E affects receptor oligomerization.[220] Some CVID patients have variants located in a highly conserved cytoplasmic domain of TACI (e.g. S231R).[219] In these patients, recruitment of MyD88 to the cytoplasmic TACI domain was disrupted, causing impaired CSR and IgG production (Figure 16).[219] Rarely, CVID patients with truncating *TNFRSF13B* variants have been reported.[217]

CVID patients with mono- or biallelic *TNFRSF13B* variants can present with a variable phenotype encompassing the complete CVID clinical spectrum (Table 3).[200,201,210-213] In some CVID-affected families, the same *TNFRSF13B* genotype has also been found in relatives with selective IgA deficiency (sIgAD) or IgG subclass deficiency (IgGSD).[200,201,212,218] Asymptomatic relatives carrying monoallelic *TNFRSF13B* variants have been shown to have *in vitro* functional B cell defects.[213]

Because of the high frequency of heterozygous *TNFRSF13B* variants in the general population, it is believed that these variants alone cannot explain the clinical phenotype in CVID patients.[210-213] Still, heterozygous *TNFRSF13B* variants can increase the risk for developing CVID by compromising B cell function and may influence the final phenotype.[220,221] TACI cooperates synergistically with TLRs in driving B cell activation and Ig production (Figure 16).[10] B cells in many CVID patients show impaired TLR7 and TLR9 responses (see section 1.5.2.5).[219] Loss-of-function *TNFRSF13B* variants might aggravate the effect of already impaired TLR signaling, or, alternatively might impose TLR signaling defects.[219] Furthermore, CVID patients with heterozygous *TNFRSF13B* variants have a higher risk of developing autoantibody-mediated autoimmunity.[209,221] In contrast, patients with biallelic *TNFRSF13B* variants seem to be protected from autoimmunity.[221]

TNFRSF13C (encoding BAFF-R) variants

Biallelic and monoallelic *TNFRSF13C* variants have been reported in about 80 CVID patients.[61,103,202,214,222-225] More than 90% of reported cases were hetero- or homozygous for the P21R missense variant.[61,103,214,222-225] In addition, other CVID patients were found to be heterozygous for H159Y, compound heterozygous for P21R and H159Y, or compound heterozygous for P21R and a complex P21R/H159Y allele.[103,222,223] One CVID patient had heterozygous P21R and H159Y variants *in cis*.[225] Interestingly, the father and sister of the last-mentioned patient also had these variants *in cis* but presented with isolated IgM deficiency respectively sIgAD.[225] Moreover, hetero- and homozygous P21R variants and heterozygous H159Y variants have also been identified in asymptomatic relatives and healthy controls.[214,222] To our knowledge, biallelic variants that include H159Y and the heterozygous P21R/H159Y allele in *TNFRSF13C* have never been reported in asymptomatic individuals.[103,223,225]

The P21R variant reduces BAFF ligand binding (Figure 16), suggestive for loss-of-function.[214] In contrast, the H159Y variant has been implicated in lymphoma development and was shown to increase TRAF recruitment and downstream BAFF-R signaling (Figure 16) when overexpressed in a HEK293 cell line, suggestive for gain-of-function.[226] Further studies will be necessary to determine the role of H159Y variants in CVID pathogenesis. The vast majority of patients with mono- or bi-allelic *TNFRSF13C* variants had normal BAFF-R protein expression.[214,222] Thus far, deficiency of BAFF-R protein expression was reported in four CVID patients.[202,224,225] Two of those BAFF-R-deficient patients were a brother and sister born from consanguineous parents with a homozygous *TNFRSF13C* 24-bp deletion causing complete loss of BAFF-R expression.[202] Furthermore, reduced (but not absent) BAFF-R expression was identified in one CVID patient with a homozygous P21R variant and the above-mentioned CVID patient with the heterozygous P21R/H159Y allele.[224,225]

Most reported CVID patients with *TNFRSF13C* variants had adulthood-onset recurrent respiratory tract infections. Nonetheless, some patients already developed symptoms at young age and/or additionally suffered from severe CVID-related complications (Table 3).[61,103,202,214,222-225] Laboratory findings varied between patients (Table 3).[61,103,202,214,222-225] Curiously, the BAFF-R-deficient sibling pair had important B cell lymphopenia with a relative increase in transitional B cells, which seems contradictory with their late disease onset and/or relatively mild clinical phenotype.[202]

Analogous to *TNFRSF13B* (TACI) variants, the role of *TNFRSF13C* (BAFF-R) variants in CVID is controversial. It is currently believed that an abnormal BAFF-R function predisposes to but does not suffice for CVID development (i.e. modifier gene).[214]

TWEAK deficiency

One CVID pedigree with autosomal dominant inheritance had a mutation in *tumor necrosis factor superfamily member 12* (*TNFSF12*), encoding TNF-like weak inducer of apoptosis (TWEAK) (Table 3).[227] TWEAK mainly exerts effects on endothelial and innate immune cells.[227] In addition to diminished TWEAK-induced signaling, mutant TWEAK associated with BAFF monomers thereby impeding BAFF-mediated signaling in B cells (Figure 16).[227] More patients will need to be identified to determine if TWEAK deficiency should be considered as a form of CVID or as a separate disorder.

B cell co-receptor complex deficiency

The B cell co-receptor complex is composed of four cell-surface proteins: CD19, CD21 (complement receptor 2, CR2), CD81 and Leu13 (Figure 16). It lowers the threshold for B cell activation upon antigen binding to the BCR.[228] CD19, CD81 and CD21 deficiencies occur in autosomal recessive forms of CVID, and were identified by use of a candidate gene approach.[228-230]

CD19 and CD81 deficiencies

Biallelic *CD19* mutations resulting in absent CD19 surface expression have been reported in seven CVID-affected families.[228,231-236] In an additional CVID patient, absent CD19 surface expression was due to a biallelic *CD81* splice site mutation.[229] This *CD81* mutation was initially assumed to completely abolish CD81 protein expression.[229] Later, it was demonstrated that in fact a truncated CD81 protein was produced.[237] Both the mutant CD81 and the normal CD19 protein were retained intracellularly, resulting in absent CD81 and CD19 surface expression.[237]

All CD19-deficient patients and the CD81-deficient patient developed symptoms in early childhood and suffered from recurrent infections.[228,229,231-236] Only the CD81-deficient patient showed autoimmune and inflammatory complications (Table 3).[229] This clinical discrepancy might be because CD81, in contrast to CD19, is involved in many immunological responses.[229] All CD19- and CD81-deficient patients had normal total $CD20^+$ B cell numbers but reduced switched memory B cells.[228,229,231-236] Impaired BCR/co-receptor complex signaling in these patients resulted in defective SHM and CSR, as well as poor terminal differentiation into memory B cells and plasma cells (see section 1.1.2.3).[238]

Interestingly, a female with isolated IgG1 deficiency was also found to have absent CD19 expression due to a homozygous *CD19* mutation.[235] She had recurrent respiratory tract infections but mainly suffered from severe IgA nephropathy. In contrast to the above-mentioned CD19-deficient CVID patients, memory B cells and responses to protein vaccines

were normal in this patient.[235] It is unclear why the latter CD19-deficient patient developed isolated IgG1 deficiency and not CVID.[235]

CD21 deficiency

Biallelic *CD21* mutations causing loss of CD21 protein expression have been published in two unrelated CVID patients.[230,239] CD19 and CD81 expression were normal. Compared to CD19- and CD81-deficient CVID patients, CD21-deficient patients demonstrated a later age of onset, milder infections and less pronounced humoral immune defects (Table 3).[230,239] Wentink *et al.* provided evidence that CD21-deficient patients can still mount proper B cell responses against antigenic stimuli but with reduced memory formation, whereas CD19- and CD81-deficient patients have a more profoundly disturbed B cell response.[239] On the other hand, CD21-deficient patients presented with chronic diarrhea, splenomegaly and/or severe myalgia, which was not seen in CD19- or CD81-deficient patients.[228-236,239]

CD20 deficiency

CD20 is part of a B cell surface complex involved in transmembrane Ca^{2+} transport, which is important in B cell signal transduction, proliferation and differentiation (Figure 16).[240] Knowledge on the exact biology of CD20 is, however, limited. CD20 is encoded by *membrane-spanning 4A1* (*MS4A1*). Using a candidate gene approach, a homozygous *MS4A1* mutation resulting in complete lack of CD20 protein expression has been identified in a single patient born from consanguineous parents.[240] She did not completely fulfill diagnostic criteria for CVID as only serum IgG was decreased with normal IgM and IgA (see section 1.3).[240] She presented with early-onset recurrent respiratory tract infections, markedly reduced class-switched memory B cells and impaired antibody responses to polysaccharide vaccines (Table 3).[240] CD20 deficiency may disrupt normal Ca^{2+} fluxing in B cells thereby compromising cell cycle progression and optimal B cell activation, which may explain the CVID-like phenotype.[240]

CD27 deficiency

CD27, a lymphocyte surface receptor encoded by *TNFRSF7*, interacts with CD70 and regulates survival, function and differentiation of T, B, NK and plasma cells (Figure 16).[241] CD27 is also used as a marker of memory B cells, like in immunophenotypical classification of CVID (see section 1.5.2.3).[242] A homozygous *TNFRSF7* mutation was first identified by targeted gene sequencing in a patient with absent CD27 protein expression.[241] So far, CD27 deficiency has been reported in 17 patients of whom 15 had homozygous *TNFRSF7* mutations (all from a consanguineous kindred) and one had a compound heterozygous

TNFRSF7 mutation.[241-243] Remarkably, in one CD27-deficient patient only a single *TNFRSF7* mutation was identified, even after extensive analysis of the entire gene locus.[242] The authors concluded that transcription of the second allele could be influenced by a mutation in a distant regulatory element or by other regulatory mechanisms.[242] The phenotype of CD27-deficient patients varied, even between those with the same genotype (Table 3).[241-243] Importantly, almost all CD27-deficient patients suffered from severe and/or atypical EBV-associated features (Table 3).[241-243] Five patients died of disease-related complications.[242] Only three of all reported patients had primary hypogammaglobulinemia initially diagnosed as CVID.[241-243] With more patients being reported, CD27 deficiency is currently considered a lymphoproliferative syndrome distinct from CVID.[242]

IL-21 and IL-21R deficiencies

IL-21 is predominantly produced by T cell subsets.[244] In contrast, IL-21 receptor (IL-21R) is widely expressed on lymphoid and myeloid cells and exerts pleiotropic immune functions.[244] Regarding humoral immunity, IL-21-IL-21R signaling is involved in GC formation, B cell differentiation and CSR, and Tfh cell development (Figure 16) (see section 1.1.2.3).[244] Biallelic loss-of-function mutations in *IL21* and *IL21R* were detected in consanguineous families using whole exome sequencing (WES) combined with either homozygosity mapping or candidate gene sequencing.[245,246] To our knowledge, one patient with IL-21 deficiency and eight with IL-21R deficiency have been published.[244-248] All IL-21(R)-deficient patients had a severe clinical presentation with high morbidity and mortality in childhood (Table 3).[244-248] IL-21(R)-deficient patients typically suffered from respiratory tract infections, inflammatory complications and/or opportunistic infections like *Cryptosporidiosis* and *Pneumocystis jirovecii* pneumonia.[244-248] Some IL-21(R)-deficient patients showed an aberrant B cell phenotype with reduced switched memory B cells. In addition, some patients demonstrated functional defects in T and NK cells (Table 3).[244-248] Several IL-21(R)-deficient patients were initially diagnosed with CVID, before the onset of opportunistic infections.[245,247] However, over time it has become evident that IL-21 and IL-21R deficiencies represent forms of CID rather than CVID.[244-248]

LRBA and CTLA-4 deficiencies

Lipopolysaccharide-responsive beige-like anchor protein (LRBA) is a cytosolic protein localized in endoplasmatic reticulum, trans-Golgi apparatus, endocytosis vesicles and lysosomes.[249] It is expressed by almost all cell types with higher expression levels in immune effector cells.[249] LRBA functions in polarized vesicle trafficking and polarized responses of immune effector cells, autophagy, and positive regulation of cell survival.[249]

CTLA-4 is an inhibitory T cell receptor that negatively regulates immune responses. It is upregulated on activated T cells and constitutively expressed by Treg cells (see also section 1.5.3.1). CTLA-4 competes with the costimulatory protein CD28 for binding to CD80/CD86, thereby preventing excessive T cell activation and maintaining immune tolerance (Figure 16).[250,251] It was recently demonstrated that LRBA plays a role in CTLA-4 surface expression.[252] LRBA is thought to rescue endosomal CTLA-4 from degradation via the lysosomal pathway and to facilitate its trafficking back to the cell surface upon TCR stimulation (Figure 16).[252]

LRBA deficiency

Biallelic loss-of-function *LRBA* mutations were identified by three independent groups by using linkage analysis in multiple consanguineous families or by using WES with or without homozygosity mapping in single consanguineous families.[249,253,254] In the vast majority of patients, biallelic *LRBA* mutations resulted in reduced or absent protein expression.[249,252-267] The clinical and immunological phenotype of LRBA-deficient patients is very variable (Table 3).[249,252-267] Most reported LRBA-deficient patients were clinically diagnosed with CVID, some with autoimmune lymphoproliferative syndrome (ALPS)-like or immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX)-like disease.[249,252-267] Typical presentations of LRBA deficiency are early-onset severe autoimmunity and enteropathy. Other recurring clinical features are recurrent infections and severe lymphoproliferative disease with increased risk of lymphoma.[249,252-267] More than half of cases had varying degrees of hypogammaglobulinemia and most patients had decreased switched memory B cells.[249,252-267] Importantly, LRBA deficiency is characterized by a progressive course and a high mortality rate.[249,252-267] Although first identified in CVID patients,[249] it is currently considered an immune dysregulation syndrome separate from CVID.[252-267]

CTLA-4 deficiency

Heterozygous mutations in *CTLA4* were identified by two independent groups using either WES combined with linkage analysis in a large family or WES combined with candidate gene sequencing.[250,251] Most *CTLA4* mutations resulted in reduced CTLA-4 expression suggesting haploinsufficiency.[250,251,268-271] Other *CTLA4* mutations were predicted to interfere with ligand binding or protein stability, which might exert a dominant-negative effect.[251] Many CTLA-4-deficient patients were clinically diagnosed with CVID.[250,251,268,269] However, *CTLA4* mutations were also detected in family members who were asymptomatic, suggesting incomplete penetrance, or who had a very mild form of antibody deficiency.[250,251,268-271] Of note, since age of onset is variable, young *CTLA4* mutation carriers who are currently asymptomatic

may still develop disease later in life.[251] Overall, the phenotype of CTLA-4 deficiency is usually severe and reminiscent of that of LRBA deficiency: autoimmunity, recurrent infections, lymphoproliferative disease with increased risk of lymphoma, and varying Ig levels and B and T cell defects (Table 3).[250,251,268-271] Treg cells were normal in numbers but had a markedly reduced suppressive function.[250,251,268,269] Treg cells of asymptomatic *CTLA4* mutation carriers also had reduced suppressive activity although they did express higher levels of CTLA-4 compared to those of their symptomatic relatives.[250,251] Analogous to LRBA deficiency, CTLA-4 deficiency was first described in CVID patients but is currently considered a new immune dysregulation syndrome.[250,251,268-271]

1.6.2.2 Genes encoding intracellular signaling molecules

Protein kinase C delta (PKCδ) is a key component in BCR-mediated signaling downstream of BTK, phospholipase C gamma 2 (PLCγ2), B-lymphoid tyrosine kinase (BLK), Vav guanine nucleotide exchange factor (VAV), and Ras-related C3 botulinum toxin substrate (RAC) (Figure 16).[272] PKCδ propagates signaling to the nucleus by activating the canonical nuclear factor of kappa light chain enhancer of activated B cells (NF-κB) pathway.[272] PKCδ is particularly important in B cell proliferation, apoptosis and tolerance.[272]

Class IA phosphatidyl-inositol-3-kinase (PI3K) isoforms are crucial signaling molecules downstream of various B and T cell surface receptors (Figure 16).[273,274] Consequently, PI3K is involved in many aspects of B and T cell homeostasis.[273,274] The PI3K pathway activates a multitude of effector molecules and is interwoven with the PLC-PKC pathway, forming a complex signaling network (Figure 16).[273,274]

The transcription factor IKAROS is a pleiotropic regulator of hematopoiesis.[275] Besides key roles in T cells and non-lymphoid lineages, IKAROS is a critical regulator of B cell lymphopoiesis and function.[275] IKAROS is triggered by (pre-)antigen receptor signaling though the precise signaling pathways remain unclear (Figure 16).[275]

Interferon regulatory factor 2 binding protein 2 (IRF2BP2) is thought to act as a negative regulator of the nuclear factor of activated T cells (NFAT) transcription factor (Figure 16).[276] In B cell biology, IRF2BP2 might play a role in the differentiation and/or survival of memory B cells and plasmablasts.[276] However, its function and interactome remain obscure.[276]

Defects in the genes encoding PKCδ, PLCγ2, NF-κB2, NF-κB1, PI3K catalytic subunit p110δ, PI3K regulatory subunit p85α, VAV1, RAC2, BLK, IKAROS and IRFBP2 have been described in CVID(-like) patients.

PKCδ deficiency

Biallelic *PRKCD* (encoding PKCδ) mutations abrogating protein expression have been described in six patients from four unrelated families.[272,277-279] *PRKCD* was detected using WES combined with homozygosity mapping or linkage analysis in consanguineous families.[272,277] PKCδ deficiency causes a variable phenotype (Table 3).[272,277-279] A CVID-like phenotype was only observed in the first-reported patient.[272] The other five patients were initially diagnosed with SLE or ALPS-like disease.[277-279] Altogether, PKCδ deficiency represents a syndrome of immune dysregulation with prominent lymphoproliferation and systemic autoimmunity reminiscent of SLE.[272,277-279] All patients displayed an aberrant B cell phenotype with decreased switched memory B cells and increased $CD21^{low}$ B cells.[272,277-279] However, only the first-reported patient developed hypogammaglobulinemia and, accordingly, prominent infections.[272] Of interest, the first-reported PKCδ-deficient patient carried an additional heterozygous *CTLA4* variant (Thr17Ala, allele frequency 0.4112), previously associated with autoimmune thyroiditis.[272] This variant was also present in the father who had Behçet's disease and autoimmune thyroiditis.[272] It cannot be excluded that this *CTLA4* variant exerted a disease-modifying effect.[272]

PLCγ2-associated antibody deficiency and immune dysregulation (PLAID)

PLCγ2-associated antibody deficiency and immune dysregulation (PLAID) is a newly defined immunodeficiency syndrome caused by heterozygous gain-of-function mutations in *PLCG2*.[280,281] *PLCG2* was identified by two independent groups, one using linkage analysis in three families combined with whole genome sequencing (WGS) in one of those families,[280] and another using WES in a multiplex family with an autosomal dominant inheritance pattern.[281] PLAID is mainly characterized by cold urticaria from infancy, which is not typically seen in CVID.[280] However, PLAID shares many hallmark clinical and immunological features with CVID (Table 3).[280,281] Indeed, some of the initially published PLAID patients fulfilled the diagnostic criteria of CVID (see section 1.3).[280] This phenotypic overlap might be explained by aberrant PLCγ2 signaling downstream of the BCR and Fcγ receptors on B cells (Figure 16).[280]

NF-κB2 and NF-κB1 deficiencies

The NF-κB family of transcription factors regulates a diversity of biological processes.[282,283] The (noncanonical) NF-κB2 pathway is activated by a limited set of receptors, including ICOS, TACI, BAFF-R and BCMA (Figure 16).[282,283] In contrast, the (canonical) NF-κB1 pathway is targeted by a vast number of receptors, including cytokine receptors, BCR/co-

receptor complex, TCR and TLRs (Figure 16).[282,283] NF-κB signaling plays key roles in B cell maturation, survival, differentiation, class switching and tolerance to self-antigens.[282,283]

NF-κB2 deficiency

First described were heterozygous *NFKB2* mutations detected by WES in a multiplex CVID pedigree with an autosomal dominant inheritance.[282] Most *NFKB2* mutations reported so far disrupted the conserved C-terminal region required for processing of the inactive precursor protein p100 into its active form p52, resulting in haploinsufficiency of NF-κB2 (also called NFKB p52/p100 subunit) (Figure 16).[282,284-289] Some *NFKB2* mutations also appeared to disrupt the canonical NF-κB1 pathway through a dominant-negative effect of the unprocessed p100 protein.[284-288] All NF-κB2-deficient patients presented with a CVID(-like) phenotype in early childhood and suffered from recurrent respiratory tract infections.[282,284-289] About half of patients developed pituitary hormone deficiencies, which is an unusual feature in CVID.[282,284-289] Two of them had pituitary hypoplasia on brain MRI scan.[284] In addition, several patients developed autoimmune manifestations involving skin, hair and/or nails. Autoimmune cytopenia, usually the predominant autoimmune manifestation in CVID, was not documented except for one child with an episode of autoimmune thrombocytopenia.[282,284-289] Furthermore, NF-κB2-deficient patients demonstrated (pan)hypogammaglobulinemia, abnormal B cell immunophenotyping and varying degrees of T cell and NK cell abnormalities (Table 3).[282,284-289]

NF-κB1 deficiency

More recently, heterozygous mutations in the gene encoding NF-κB1 (also known as NFKB p50/p105 subunit) were uncovered by means of WES combined with linkage analysis in a large CVID-affected family with autosomal dominant inheritance (Figure 16).[283] In total, heterozygous *NFKB1* mutations have been reported in 10 families of which 9 harbored CVID patients.[283,290-293] All mutant *NFKB1* alleles resulted in loss-of-function, the larger part through haploinsufficiency and some by a dominant-negative effect.[283,290-293] In contrast to *NFKB2*, *NFKB1* mutations were also identified in family members who were asymptomatic, suggestive of incomplete penetrance, or who displayed milder forms of antibody deficiency.[283,290-293] NF-κB1-deficient patients displayed a variable age of onset and a clinical phenotype different from that of NF-κB2-deficient patients (Table 3).[283,290-293] Although most NF-κB1-deficient patients also had recurrent infections, autoimmune manifestations did not usually involve hair, skin or nails, but rather blood cells, thyroid gland and other organs (Table 3).[283,290-293] Moreover, several patients suffered from autoinflammatory complications

such as enteropathy or Behçet-like aphthous ulcers (Table 3).[283,290-293] Interestingly, polymorphisms in *NFKB1* had been previously associated with inflammatory bowel disease and Behçet disease.[294,295] None of the NF-κB1-deficient patients had pituitary hormone deficiencies.[283,290-293] The majority of cases had varying degrees of antibody deficiency, though not always fulfilling CVID criteria. B, T and NK cell abnormalities also differed among patients (Table 3).[283,290-293]

PI3K overactivity and deficiency

PI3K p110δ overactivity

A heterozygous mutation in the gene encoding the PI3K catalytic subunit p110δ (*PIK3CD*) was initially identified in a CVID patient in 2006 based on a mouse knockout model.[296] Since 2013, heterozygous *PIK3CD* mutations have been described in almost 50 families using WES.[273,274,297-304] These mutations result in overactivity of the PI3K signaling pathway evidenced by enhanced p110δ membrane association and kinase activity.[273,274] More than half of reported families were heterozygous for the missense mutation E1021K.[273,274,296-304] Haplotype analysis was suggestive for a recurrent rather than for a founder mutation.[273] PI3K p110δ mutant patients showed phenotypic overlap with many other PID syndromes; the majority was diagnosed with CVID, CID or hyper-IgM syndrome.[273,274,296-304] Therefore, the phenotype associated with dominant *PIK3CD* gain-of-function mutations was regarded as a novel disease named activated PI3Kδ syndrome (APDS).[273] The clinical spectrum of APDS varies greatly (Table 3).[273,274,296-304] Important is the increased risk of malignancy (mainly B cell lymphoma) even in patients with a seemingly milder phenotype.[273,274,296-304] The constitutively activated PI3K pathway caused numerous defects in B and T cell differentiation and function (Table 3).[273,274,296-304] Recurring features were increased serum IgM, decreased serum IgG and IgA, progressively decreasing B cells, expansion of transitional B cells, $CD4^+$ T cell lymphopenia, and elevated $CD8^+$ effector memory cells.[302]

PI3K p85α deficiency

Some patients with an APDS-like phenotype were found to have heterozygous loss-of-function mutations in *PIK3R1*, encoding the PI3K regulatory subunit p85α, by means of WES.[305-311] In particular, all mutations were located within the splice acceptor or donor site of exon 11 of the *PIK3R1* gene, causing in-frame skipping of that exon and formation of a shortened p85α protein.[305-311] Exon 11 forms part of the domain that inhibits p110δ catalytic activity.[311] Loss of p85α-mediated inhibition of p110δ causes hyperactivity of the PI3Kδ signaling pathway in B and T lymphocytes (Figure 16), explaining the APDS-like phenotype

seen in these patients (Table 3).[305-311] Accordingly, this disease entity is referred to as APDS2.[311] As for patients with *PIK3CD* gain-of-function mutations (APDS1), the major complication in APDS2 is development of B cell lymphoma.[311] Since all APDS2 patients had recurrent respiratory tract infections and more than 85% had decreased serum IgG and IgA, it is not surprising that many were previously diagnosed with CVID (Table 3).[311]

Noteworthy, a homozygous *PIK3R1* mutation resulting in a premature stop codon in exon 6 and complete loss of p85α expression had been previously reported in a single patient born from consanguineous parents.[312] In contrast to the above-described heterozygous splice site mutations, complete loss of p85α resulted in a significant reduction of PI3K signaling causing agammaglobulinemia and absence of B cells.[312] Furthermore, heterozygous *PIK3R1* mutations downstream of exon 11 are known to cause SHORT syndrome associated with loss of PI3K activity.[310,311,313] This syndrome is, among others, characterized by progeroid facial dysmorphism, short stature and in some cases immunodeficiency.[313] Short stature was also observed in about 45% of patients with the above-mentioned *PIK3R1* splice site mutations, and microcephaly in about 10% (Table 3).[309,311] In conclusion, three different types of genetic defects affecting other sites of *PIK3R1* give rise to three distinct phenotypes (APDS2, agammaglobulinemia and SHORT syndrome), suggesting a certain degree of genotype-phenotype correlation for *PIK3R1* mutations.

VAV1, RAC2 and BLK deficiencies

A heterozygous *Vav1 guanine nucleotide exchange factor* (*VAV1*) mutation resulting in decreased protein expression was described in one patient diagnosed with CVID.[314] Since this patient showed considerable T cell dysfunction (Table 3), VAV1 deficiency more likely causes CID rather than CVID.[314]

A homozygous *Ras-related C3 botulinum toxin substrate 2* (*RAC2*) mutation abolishing protein expression was identified in two siblings born from consanguineous parents.[315] Both siblings presented with IgA deficiency that gradually evolved into CVID (Table 3).[315] Interestingly, heterozygous dominant-negative *RAC2* mutations cause a complex neutrophil dysfunction.[315] The RAC2-deficient CVID patients showed less severe defects in neutrophil function.[315]

A heterozygous loss-of-function mutation in *B-lymphoid tyrosine kinase* (*BLK*) was detected in two related CVID patients.[316] BLK plays a role in BCR signaling and recruitment of T cell help (Figure 16).[316] This may explain the disturbed terminal B cell differentiation seen in these patients (Table 3).[316]

More patients will need to be identified to determine if VAV1, RAC2 and BLK deficiencies should be considered as CVID or as separate disease entities.

IKAROS deficiency

A heterozygous mutation in *IKZF1*, encoding the transcription factor IKAROS, was first described in 2012 in a premature infant with fetal hydrops, pancytopenia and absent myelopoiesis who died of multi-organ failure after bone marrow transplantation.[317] In 2016, the same mutation as well as 10 other heterozygous mutations involving *IKZF1,* were identified in 12 families affected with CVID or other forms of antibody deficiency.[275,318] All mutations disrupt the DNA-binding domain of IKAROS, resulting in failure to bind target genes and haploinsufficiency (Figure 16).[275,317,318] Incomplete penetrance was documented in about half of the currently reported families.[275,318] Some asymptomatic individuals were children, however, who might still develop a clinical phenotype at older age.[275,318] Manifesting IKAROS-deficient patients had a variable phenotype (Table 3).[275,317,318] They mainly suffered from recurrent respiratory tract infections, four families had autoimmune manifestations, and acute leukemia was seen in two families (Table 3).[275,317,318] Patients generally presented with severe B cell lymphopenia accompanied by a decrease in at least one major immunoglobulin isotype (Table 3).[275,317,318] Moreover, disease course was seen to be progressive, with loss of B cells and serum immunoglobulins over time.[275,318] The T cell compartment appeared less severely affected, although various alterations in peripheral T cell subsets were reported (Table 3).[275,317,318] Since not all *IKZF1* mutant patients developed a CVID phenotype, this disease entity is better referred to as IKAROS deficiency (Table 3).[275,317,318]

IRF2BP2 overactivity

Very recently, one family with an autosomal dominant pattern of CVID was identified with a heterozygous *IRF2BP2* mutation cosegregating with disease.[276] *In vitro* analyses demonstrated that the *IRF2BP2* mutation impaired plasmablast differentiation of B cells.[276] Furthermore, subjects with the heterozygous *IRF2BP2* mutation had increased levels of the corresponding transcripts and protein.[276] The findings are suggestive for a gain-of-function mutation and for an augmented repression of NFAT transcriptional activity by IRF2BP2 (Figure 16).[276] However, additional studies are needed to uncover the mechanism by which the *IRF2BP2* mutation disturbs B cell biology and causes a CVID phenotype.[276] All patients with the heterozygous *IRF2BP2* mutation were diagnosed with CVID in childhood. The main phenotypical features were recurrent sinopulmonary infections, decreased IgG (mainly IgG2), low to undetectable IgA and IgM levels, and very low memory B cells (Table 3).[276] They did not display evidence of T cell dysfunction.[276]

Table 3. Genes associated with monogenic forms of CVID: overview of genetic, clinical and immunological characteristics.

Gene, OMIM number	Number of publ. patients	Effect on protein	Allelicity	Onset	Clinical spectrum	Immunological spectrum	CVID or separate entity	Ref
Genes encoding receptors and ligands								
***ICOS*, *604558**	15 (7 fam.)	LOF (absent expr.)	Biallelic	Infancy to adulthood	RTI, GI infections, opportunistic infections, bacterial skin infections, localized HSV infections, neuroborreliosis, bronchiectasis, AI (incl. AI cytopenia, AI arthritis), BLH, splenomegaly, hepatomegaly, granulomata, enteropathy, malignancy.	↓ IgG, ↓ or nl IgM, ↓ or nl IgA, ↓ antibody responses to protein and/or polysaccharide vaccines, ↓ or nl total B cells, ↓ memory B cells, ↓ bone marrow plasma cells, nl total/$CD4^+$/ $CD8^+$ T cells, ↓ or nl $CD4^+$ and $CD8^+$ memory T cells, nl Treg cells, ↓ or nl circulating Tfh cells, ↓ or nl production of Th1/Th2/Th17 cytokines, ↓ CTLA-4 expr, nl CD40(L) expr.	ICOS deficiency	199, 204-208
***TNFRSF13B* (TACI), *604907**	2147	LOF (usually nl expr.)	Monoallelic/ biallelic	Early childhood to adulthood	RTI, GI infections, bronchiectasis, AI (incl. AI cytopenia, AI arthritis), BLH, splenomegaly (+/- splenectomy), granulomata, enteropathy, malignancy. Note: variants also found in asymptomatic individuals and in patients with IgGSD or sIgAD.	↓ IgG, ↓ or nl IgM, ↓ or nl IgA, ↓ antibody responses to polysaccharide vaccines, ↓ or nl or ↑ total B cells, ↓ or nl memory B cells, ↓ or nl total/$CD4^+$/ $CD8^+$ T cells, ↓ or nl $CD4^+$ and $CD8^+$ naive/memory T cells, ↓ or nl Treg cells.	CVID, disease-predis-posing	103, 200, 201, 210-213, 215-221
***TNFRSF13C* (BAFF-R), *606269**	> 80	LOF/GOF (usually nl expr.)	Monoallelic/ biallelic	Infancy to late adulthood	RTI, GI infections, cholangitis, sacroiliitis, bronchiectasis, AI (incl. AI cytopenia), BLH, splenomegaly, granulomata, enteropathy, failure to thrive. Note: variants also found in asymptomatic individuals and in patients with sIgAD or sIgMD.	↓ IgG, nl to undetectable IgM, nl to undetectable IgA, ↓ antibody responses to polysaccharide vaccines, ↓ or nl total B cells, nl or ↑ transitional B cells, nl or ↓ memory B cells, nl total T cells, nl T cell subsets.	CVID, disease-predis-posing	61, 103, 202, 214, 222-225
***TNFSF12* (TWEAK), *602695**	3 (1 fam.)	LOF (nl expr.)	Monoallelic	Infancy	RTI, pneumococcal meningitis, osteomyelitis, AI thrombocytopenia and neutropenia, warts.	↓ IgG or low nl IgG with ↓ IgG2, ↓ IgM, ↓ IgA, ↓ antibody responses to protein and polysaccharide vaccines, ↓ or nl total B cells, ↓ memory B cells, ↑ naive B cells, nl or ↑ total T cells, nl total $CD4^+$ T cells, ↑ total $CD8^+$ T cells, ↑ double negative T cells, ↓ *in vitro* apoptotic function.	CVID	227
***CD19*, *607265**	10 (7 fam.)	LOF (↓ or absent expr.)	Biallelic	Infancy to early childhood	RTI, GI infections, bacterial skin and soft tissue infections, bronchiectasis, intermittent microscopic hematuria, postinfectious glomerulonephritis, IgA nephropathy.	↓ IgG, ↓ or nl IgM, ↓ or nl IgA, ↓ antibody responses to protein and polysaccharide vaccines, nl total $CD20^+$ B cells, ↓ memory B cells, ↓ BCR signaling, nl CD81 expr., ↓ CD21 expr., nl total T cells, nl T cell subsets.	CVID	228, 231-236

Continued

Table 3. Continued.

***CD81*, *186845**	1	LOF (absent expr.)	Biallelic	Infancy	RTI, AI thrombocytopenia, severe glomerulonephritis with progression to end-stage renal disease, undefined systemic inflammatory syndrome.	↓ IgG, nl IgM, ↓ to low nl IgA, ↓ antibody responses to protein and polysaccharide vaccines, nl total $CD20^+$ B cells, ↓ memory B cells, ↓ BCR signaling, absent CD19 expr., ↓ CD21 expr., nl total T cells, nl T cell subsets.	CVID	229, 237
***CR2* (CD21), *120650**	2 (2 fam.)	LOF (absent expr.)	Biallelic	Early childhood to childhood	RTI, enteropathy, splenomegaly, myalgia, rigidity.	↓ IgG, ↓ or nl IgM, ↓ IgA, ↓ antibody response to polysaccharide vaccines, nl total $CD19^+$ B cells, ↓ memory B cells, mildly ↓ BCR signaling, nl CD19/CD81 expr., nl total T cells, nl T cell subsets.	CVID	230, 239
***MS4A1* (CD20), *112210**	1	LOF (absent expr.)	Biallelic	Infancy	RTI.	↓ IgG, nl IgM, nl IgA, ↓ antibody responses to polysaccharide vaccines, nl total B cells, ↓ memory B cells, nl total T cells, nl T cell subsets.	CVID	240
***TNFRSF7* (CD27), *186711**	17 (9 fam.)	LOF (↓ or absent expr.)	Biallelic	Infancy to childhood	Chronic EBV viremia, severe/atypical EBV-associated infections (e.g. severe mononucleosis, pneumonia, meningitis/encephalitis, oral/perianal ulcers, uveitis), EBV-induced lymphoproliferation (incl. BLH, splenomegaly, hepatomegaly, lymphocytic infiltration of non-lymphoid organs, HLH, lymphoma), RTI, bronchiectasis, bacterial skin infections, giardiasis, fulminant bacterial sepsis.	↓ or nl or ↑ IgG, ↓ or nl IgM, ↓ or nl IgA, ↓ or nl antibody responses to protein and/or polysaccharide vaccines, ↓ or nl total B cells, ↓ memory B cells, nl or ↑ transitional B cells, nl or ↑ $CD21^{low}$ B cells, ↓ or nl $CD4^+$ T cells, nl or ↑ $CD8^+$ T cells, ↓ or nl $CD8^+$ memory T cells, ↓ or nl *in vitro* T cell proliferation responses, ↓ or nl or ↑ NK cells, ↓ or nl NK cell cytotoxicity, ↓ or nl iNKT cells.	CD27 deficiency	241-243
***IL21*, *605384**	1	LOF (nl expr.)	Biallelic	Infancy	RTI, early-onset IBD, failure to thrive, recurrent oral aphthous ulcers.	↓ IgG, nl IgM, nl IgA, ↑ IgE, ↓ antibody responses to protein and polysaccharide vaccines, ↓ total B cells, ↓ memory B cells, ↓ naive B cells, ↑ transitional B cells, nl total/$CD4^+$/$CD8^+$ T cells, ↓ *in vitro* T cell proliferation responses.	IL-21 deficiency	245
***IL21R*, *605383**	8 (6 fam.)	LOF (↓ or absent expr.)	Biallelic	Infancy to early childhood	RTI, GI infections, opportunistic infections (incl. cryptosporidiosis with progression to end-stage biliary/liver disease), pulmonary tuberculosis, bronchiectasis, BLH, hepatosplenomegaly, discoid lupus/chronic inflammatory skin disease, failure to thrive.	↓ or nl IgG, nl or ↑ IgM, ↓ or nl IgA, nl or ↑ IgE, ↓ antibody responses to protein and/or polysaccharide vaccines, ↓ or nl or ↑ total B cells, ↓ or nl memory B cells, nl or ↑ transitional B cells, nl total T cells, ↓ or nl $CD4^+$ T cells, ↓ or nl $CD8^+$ T cells, ↓ or nl Tfh cells, ↓ or nl *in vitro* B and T cell proliferation responses, ↓ or nl production of Th cytokines, ↓ or nl NK cells, ↓ or nl NK cell cytotoxicity.	IL-21R deficiency	244, 246-248

Continued

Table 3. Continued.

***LRBA*, *606453**	62 (44 fam.)	LOF (majority ↓ or absent expr.)	Biallelic	Infancy to childhood	Severe AI (incl. AI cytopenia, type 1 diabetes mellitus, AI thyroid disease, AI adrenal insufficiency, vitiligo, AI arthritis), severe (EBV-induced) lymphoproliferation with generalized BLH and lymphocytic infiltration of organs (e.g. kidney, brain), LIP, GLILD, granulomata, severe enteropathy, chronic lung disease, bronchiectasis, splenomegaly, hepatomegaly, malignancy, finger clubbing, failure to thrive, RTI, GI infections, opportunistic infections, bacterial skin and soft tissue infections, deep abscesses, warts, localized mollusca contagiosa, food allergy, allergic dermatitis, urticaria, growth hormone deficiency.	↓ or nl or ↑ IgG, ↓ or nl IgM, ↓ or nl or ↑ IgA, ↓ or nl antibody responses to protein and/or polysaccharide vaccines, ↓ or nl total lymphocytes, ↓ or nl total B cells, ↓ or nl memory B cells, ↓ or nl plasmablasts, ↓ or nl or ↑ transitional B cells, ↓ or nl or ↑ naive B cells, nl or ↑ $CD21^{low}$ B cells, ↓ B cell proliferation and Ig secretion, ↓ or nl or ↑ total/$CD4^+$/$CD8^+$ T cells, ↓ or nl $CD4^+$/$CD8^+$ naive T cells, nl or ↑ $CD4^+$/$CD8^+$ memory T cells, ↓ or nl Treg cells, nl or ↑ DN T cells, nl or ↑ Tfh cells, ↓ or nl *in vitro* T cell proliferation responses, ↓ or nl Fas-mediated apoptosis, ↓ CTLA-4 surface expr., ↑ sCD25, ↓ or nl NK cells, ↓ or nl neutrophils.	LRBA deficiency	249, 252-267
***CTLA4*, *123890**	32 (19 fam.)	LOF (usually ↓ expr.)	Monoallelic	Infancy to adulthood	Severe AI (incl. AI cytopenia, type 1 diabetes mellitus, AI thyroid disease, AI arthritis), severe (EBV-induced) lymphoproliferation with generalized BLH and lymphocytic infiltration of organs (e.g. kidney, brain, bone marrow), GLILD, granulomata, severe enteropathy, bronchiectasis, splenomegaly, hepatomegaly, malignancy (mainly lymphoma), failure to thrive, RTI, GI infections, opportunistic infections, pulmonary tuberculosis, warts, food allergy, allergic dermatitis. Note: variants also found in asymptomatic individuals.	↓ or nl IgG, ↓ or nl IgM, ↓ or nl IgA, ↓ or nl antibody responses to polysaccharide vaccines, ↓ or nl total lymphocytes, ↓↓ or ↓ or nl total B cells, ↓ or nl memory B cells, nl or ↑ $CD21^{low}$ B cells, ↓ or nl total T cells, ↓ or nl $CD4^+$/$CD8^+$ T cells, ↓↓ or ↓ or nl $CD4^+$/$CD8^+$ naive T cells, nl or ↑ double negative T cells, nl or ↑ Treg cells, ↓ FoxP3/CD25 expr. on Treg cells, ↓ suppressive activity Treg cells, ↑ activity effector T cells, ↓ or nl NK cells, ↓ or nl NKT cells.	CTLA-4 deficiency	250, 251, 268-271
Genes encoding intracellular signaling molecules								
***PRKCD*, *176977**	6 (4 fam.)	LOF (↓ or absent expr.)	Biallelic	Infancy to early childhood	Severe systemic AI with features reminiscent of SLE, severe (EBV/CMV-induced) lymphoproliferation with generalized BLH, splenomegaly, hepatomegaly, RTI, GI infections, urinary tract infections, failure to thrive.	↓ or nl IgG, nl or ↑ IgM, nl or ↑ IgA, ↓ or nl antibody responses to protein and/or polysaccharide vaccines, ↓ or nl or ↑ total B cells, ↓ memory B cells, nl or ↑ transitional B cells, nl or ↑ naive B cells, ↑ $CD21^{low}$ B cells, ↓ or nl total/$CD4^+$/$CD8^+$ T cells, nl or ↑ double negative T cells, mildly ↓ or nl *in vitro* T cell proliferation responses, ↓ or nl NK cells, ↓ or nl NK cell cytotoxicity, nl NKT cells, ↓ or nl neutrophil microbial killing capacity.	PKCδ deficiency	272, 277-279

Continued

Table 3. Continued.

***PLCG2*, *600220**	30 (4 fam.)	GOF (usually nl expr.)	Monoallelic	Infancy to childhood	Cold urticaria (negative ice cube skin test, positive evaporative cooling skin test), atopy (food, airway, skin), skin granulomata, blistering skin lesions, RTI, onychomycosis, VZV infections, bacterial skin infections, AI (mainly involving skin and thyroid gland).	↓ or nl IgG, ↓ or nl IgM, ↓ or nl IgA, nl or ↑ IgE, ↓ or nl antibody responses to polysaccharide vaccines, nl total B cells, ↓ or nl memory B cells, ↓ BCR signaling, ↓ *in vitro* B cell proliferation responses, negative cold agglutinins and cryoglobulins, positive anti-nuclear antibodies, nl total T cells, nl T cell subsets, ↓ or nl NK cells, ↓ or nl NKT cells.	PLAID	280, 281
***NFKB2*, *164012**	18 (11 fam.)	LOF (↓ or nl expr.)	Monoallelic	Infancy to childhood	RTI, GI infections, localized HSV infections, oral candidiasis, onychomycosis, bronchiectasis, pituitary hormone deficiencies (mainly ACTH deficiency), AI (mainly involving skin, hair and nails).	↓ or nl IgG, ↓ or nl IgM, ↓ or nl IgA, ↓ or nl antibody responses to protein and/or polysaccharide vaccines, ↓ or nl B cells, ↓ or nl memory B cells, nl or ↑ total T cells, nl $CD4^+/CD8^+$ T cells, ↓ or nl $CD4^+/CD8^+$ memory T cells, nl or ↑ recent thymic emigrant $CD4^+$ T cells, ↓ or nl Treg cells, ↓ or nl circulating Tfh cells, ↓ or nl *in vitro* T cell proliferation responses, ↓ or nl NK cells, ↓ or nl NK cell cytotoxicity.	NF-κB2 deficiency	282, 284-289
***NFKB1*, *164011**	35 (10 fam.)	LOF (↓ or nl expr.)	Monoallelic	Early childhood to adulthood	RTI, GI infections, bacterial skin and soft tissue infections, deep abscesses, localized HSV / VZV infections, genital candidiasis, AI (incl. AI cytopenia, alopecia, AI thyroid disease, AI arthritis), pyoderma gangrenosum, Behçet-like disease, enteropathy, bronchiectasis, chronic lung disease, (EBV-induced) lymphoproliferation with (generalized) BLH, LIP, splenomegaly, hepatomegaly, gastric adenoma, malignancy. Note: variants also found in asymptomatic individuals and in patients with other antibody deficiencies (e.g. IgGSD, sIgAD).	Full immunol. phenotype not reported in ref [283]. ↓ or nl IgG, ↓ or nl IgM, ↓ or nl IgA, ↓ or nl antibody responses to protein and/or polysaccharide vaccines, ↓ or nl total B cells, ↓ or nl or ↑ memory B cells, ↓ or nl or ↑ transitional B cells, ↓ or nl or ↑ naive B cells, nl or ↑ $CD21^{low}$ B cells, nl total T cells, nl $CD4^+/CD8^+$ T cells, ↓ or nl or ↑ $CD4^+/CD8^+$ naive/memory T cells, ↓ or nl Treg cells, nl or ↑ DN T cells, nl or ↑ γδ T cells, ↓ or nl *in vitro* T cell proliferation responses, ↓ or nl NK cells.	NF-κB1 deficiency	283, 290-293

Continued

Table 3. Continued.

***PIK3CD*, *602839**	88 (49 fam.)	GOF (usually nl expr.)	Monoallelic	Infancy to early childhood	RTI, GI infections, bacterial skin and soft tissue infections, deep abscesses, persistent CMV / EBV viremia, recurrent HSV infections, severe VZV infections, warts, localized mollusca contagiosa, mucocutaneous candidiasis, failure to thrive, bronchiectasis, AI (incl. AI cytopenia, AI primary sclerosing cholangitis, AI glomerulonephritis, AI thyroid disease), enteropathy, (EBV/CMV-induced) lymphoproliferation with (generalized) BLH and lymphocytic infiltration of organs (e.g. gut), splenomegaly, hepatomegaly, malignancy (mainly lymphoma), mild neuro-developmental delay.	↓ or nl or ↑ IgG, ↓ or nl IgG2, ↓ or nl or ↑ IgA, ↓ or nl or ↑ IgM, ↓ or nl antibody responses to protein and/or polysaccharide vaccines, ↓ or nl total lymphocytes, ↓ or nl total B cells, ↓ or nl memory B cells, nl or ↑ transitional B cells, ↓ or nl naive B cells, ↓ or nl or ↑ total/ $CD4^+$/ $CD8^+$ T cells, ↓ or nl $CD4^+$/$CD8^+$ naive T cells, nl or ↑ $CD4^+$/$CD8^+$ memory T cells, ↓ or nl recent thymic emigrant $CD4^+$ T cells, nl Treg cells, ↓ or nl circulating Tfh cells, ↑ T cell activation-induced cell death, ↓ or nl or ↑ NK cells, ↓ or nl or ↑ NKT cells, ↓ or nl NK cell cytotoxicity.	APDS (APDS1)	273, 274, 296-304
***PIK3R1*, *171833**	47 (42 fam.)	LOF (nl expr.)	Monoallelic	Infancy to childhood	RTI, GI infections, bacterial skin and soft tissue infections, persistent CMV / EBV viremia, chronic HBV / HCV hepatitis, severe VZV infections, warts, localized mollusca contagiosa, mucocutaneous candidiasis, failure to thrive, bronchiectasis, AI (incl. AI cytopenia, AI arthritis, type 1 diabetes mellitus, AI hepatitis), enteropathy, (EBV/CMV-induced) lymphoproliferation with (generalized) BLH and lymphocytic infiltration of organs (e.g. gut), splenomegaly (+/- splenectomy), hepatomegaly, malignancy (mainly lymphoma), short stature, mild neuro-developmental delay, microcephaly.	↓ or nl or ↑ IgG, ↓ or nl or ↑ IgM, ↓ or nl or ↑ IgA, ↓ antibody responses to polysaccharide vaccines, ↓ or nl total lymphocytes, ↓ or nl total B cells, ↓ or nl memory B cells, nl or ↑ transitional B cells, nl or ↑ $CD21^{low}$ B cells, nl or ↑ total T cells, ↓ or nl $CD4^+$ (total/naive/memory) T cells, ↓ or nl or ↑ $CD8^+$ (total/naive/memory) T cells, nl or ↑ Treg cells, nl DN T cells, ↓ Th17 cells, ↓ or nl *in vitro* T cell proliferation responses, ↓ or nl or ↑ NK cells.	APDS-like (APDS2)	305-311
***VAV1*, *164875**	1	LOF (↓ expr.)	Monoallelic	Adulthood	Full clinical phenotype not reported. RTI, GI infections, genitourinary infections, bronchiectasis.	Full immunological phenotype not reported. ↓ IgG, ↓ IgM, ↓ IgA, nl total B cells, nl total T cells, ↓ $CD4^+$ T cells, nl $CD8^+$ T cells, ↓ *in vitro* T cell proliferation responses to mitogens.	Vav1 deficiency	314
***RAC2*, *602049**	2 (1 fam.)	LOF (absent expr.)	Biallelic	Infancy to childhood	RTI, failure to thrive, bronchiectasis, arthralgia, AI endocrinopathy, BLH, poststreptococcal glomerulonephritis (+/-progression to end-stage renal disease), solar urticaria, food allergy, coagulopathy.	↓ IgG, ↓ IgM, ↓ IgA, ↓ antibody responses to polysaccharide vaccines, ↓↓ total B cells, nl total/$CD4^+$/$CD8^+$ T cells, ↓ $CD4^+$/$CD8^+$ naive T cells, ↓ recent thymic emigrant $CD4^+$ T cells, ↓ Treg cells, ↓ TRECs, ↓ KRECs, nl neutrophils, ↓ neutrophil chemotaxis, ↓ & aberrant morphology of neutrophil granules.	RAC2 deficiency	315

Continued

Table 3. Continued.

***BLK*, *191305**	2 (1 fam.)	LOF (nl expr.)	Monoallelic	Infancy	RTI, bacterial skin infections.	↓ IgG, ↓ or nl IgA, ↓ or nl IgM, ↓ antibody responses to polysaccharide vaccines, ↓ or nl total B cells, nl total T cells.	CVID	316
***IKZF1*, *603023**	31 (13 fam.)	LOF (↓ or nl expr.)	Monoallelic	Early childhood to late adulthood	RTI, GI infections, bacterial skin infections, opportunistic infections, AI (incl. AI cytopenia, SLE, IgA vasculitis), malignancy (acute lymphoblastic leukemia), premature birth. Note: variants also found in asymptomatic individuals.	↓ IgG, ↓ or nl IgM, ↓ or nl IgA, ↓ or nl antibody responses to protein and/or polysaccharide vaccines, ↓ or nl total B cells, ↓ or nl memory B cells, nl or ↑ total T cells, ↓ or nl or ↑ $CD4^+$/$CD8^+$ T cells, ↓ or nl naive $CD4^+$/$CD8^+$ T cells, ↓ or nl memory $CD4^+$/$CD8^+$ T cells, ↓ or nl Treg cells, nl or ↑ circulating Tfh cells, nl or ↑ DN T cells, ↓ or nl γδ T cells, ↓ or nl *in vitro* T cell proliferation responses, nl Fas-mediated apoptosis, ↓ or nl or ↑ NK cells, ↓ or nl NK cell cytotoxicity, ↓ or nl NKT cells, pancytopenia, bone marrow abnormalities (↓ common lymphoid progenitor cells, ↓ overall myelopoiesis).	IKAROS deficiency	275, 317, 318
***IRF2BP2*, *615332**	3 (1 fam.)	GOF (↑ expr.)	Monoallelic	Early childhood to childhood	RTI, AI (incl. type 1 diabetes mellitus, psoriasis), enteropathy.	↓ IgG, ↓ IgG2, ↓ to undetectable IgM, undetectable IgA, ↓ antibody responses to protein and/or polysaccharide vaccines, nl total B cells, ↓ memory B cells, nl total/$CD4^+$/$CD8^+$ T cells, ↓ or nl NK cells.	CVID	276

Disease onset: infancy (0-2 years), early childhood (3-8 years), childhood (9-17 years), adulthood (18-50 years), late adulthood (>50 years).

↓: decreased, ↑: increased, +/-: with or without, ACTH: adrenocorticotropic hormone, AI: autoimmun(e)(ity), APDS: activated PI3 kinase delta syndrome, BCR: B cell receptor, BLH: benign lymphoid hyperplasia, CMV: cytomegalovirus, CVID: common variable immunodeficiency, DN T: double negative T ($CD4^-CD8^-$), EBV: Epstein-Barr virus, expr.: expression, fam.: families, GI: gastro-intestinal, GLILD: granulomatous lymphocytic interstitial lung disease, GOF: gain-of-function, HLH: hemophagocytic lymphohistiocytosis, HBV: hepatitis B virus, HCV: hepatitis C virus, HSV: herpes simplex virus, IgGSD: IgG subclass deficiency, incl.: including, KRECs: κ-deleting recombination excision circles, LIP: lymphoid interstitial pneumonia, LOF: loss-of-function, nl: normal, PLAID: PLCγ2-associated antibody deficiency and immune dysregulation, publ.: published, ref: references, RTI: respiratory tract infections, sIgAD: selective IgA deficiency, sIgMD: selective IgM deficiency, SLE: systemic lupus erythematosus, Tfh: follicular helper T ($CD4^+CD45RO^+CXCR5^+$), Th: T helper ($CD4^+$), TRECs: T cell receptor excision circles, Treg: regulatory T ($CD4^+CD25^+FoxP3^+$), VZV: varicella zoster virus.

1.6.2.3 Genes associated with other PID disorders

The first disease stages of other PID disorders may sometimes resemble CVID. In addition, a large number of well-defined PIDs can be accompanied by CVID-reminiscent antibody deficiency.[20] Mutations in *GATA2*, *STAT1*, *PTEN*, *RAG1*, *IL2RG*, *JAK3* and *DCLRE1C* (encoding ARTEMIS), genes associated with other well-defined PID syndromes, were recently described in patients with a prior diagnosis of CVID.[304,319-326]

Monoallelic loss-of-function mutations in *GATA2* cause a PID syndrome typically characterized by decreased monocytes, B cells, NK cells and DCs, myelodysplasia, opportunistic infections and lymphedema.[319] In a recent report, a boy with a heterozygous *GATA2* mutation presented with hypogammaglobulinemia and defective antibody responses in early childhood diagnosed as CVID.[319] However, during adolescence his monocyte and lymphocyte counts rapidly dropped resulting in a full-blown GATA2 deficiency syndrome.[319]

Heterozygous *STAT1* gain-of-function mutations underlie chronic mucocutaneous candidiasis as well as a wide range of other clinical features.[327] A 10-year-old boy was diagnosed with CVID because of low serum immunoglobulins, impaired vaccine responses, low B cells and recurrent respiratory tract infections, despite multiple bouts of mucocutaneous candidiasis since early childhood.[320] WES was required to reach a conclusive diagnosis of STAT1 gain-of-function syndrome.[320]

Autosomal dominant loss-of-function mutations in *PTEN* cause a rare familial cancer syndrome known as *PTEN* hamartoma tumor syndrome or Cowden syndrome. In addition to multiple hamartomas and an increased risk of developing certain types of cancer, some patients demonstrate defects of the adaptive immune system.[328] Recently, there have been several case series on patients with *PTEN* deficiency presenting varying degrees of antibody deficiency, autoimmunity and lymphoproliferative disease. Given the phenotypical overlap, some of these patients had received a prior diagnosis of CVID or APDS.[304,321,329]

Bilallelic loss-of-function mutations in *RAG1*/*RAG2, IL2RG*, *JAK3* and *DCLRE1C* are historically associated with severe combined immune deficiency, but hypomorphic mutations are known to cause a more insidious clinical picture.[322-326] Occasionally, patients can present with early-onset antibody deficiency, impaired vaccine responses and (sub)normal lymphocyte counts, eliciting a diagnosis of CVID as demonstrated in recent publications.[322-326]

1.6.3 Complex forms of CVID

1.6.3.1 CVID, a complex disorder?

It is increasingly believed that besides rare monogenic forms, CVID is a polygenic or multifactorial disorder. This is based on the following: (1) identification of pathogenic mutations in only 2-10% of CVID patients despite tremendous efforts, (2) large phenotypic variability between patients with the same primary genotype, (3) presence of variants in asymptomatic relatives and/or in the general population above a certain threshold frequency, (4) sporadic occurrence in about 90% of cases, and (5) delayed disease onset in many patients.[198,203,211,222,250,251,283,330-332]

Widespread use of NGS technologies has fueled the idea of a possible polygenic nature of CVID.[198,203,330,331] van Schouwenburg *et al.* performed WGS in 32 sporadic CVID patients and one grandmother-grandson pair combined with RNA sequencing of B cells in three sporadic patients.[198] They observed that all patients had variants in multiple genes associated with CVID or other PID syndromes as well as an enrichment of variants in pathways important in B cell function.[198] An average of 9.4 (range 5-15) variants possibly associated with CVID were found in each patient.[198] Interestingly, predicted deleterious variants were identified in numerous genes not previously associated with CVID such as *PRRC2A*, *LILRB5*, *PSMB9*, *TNIP1*, *ARID3A*, *INPP5D*, *SH3BP2*, *BANK1*, *GAB2*, *CAMLG*, *BCL2L11* and *EBF1*.[198] Note that functional validation studies are still necessary to determine the contribution of these variants to CVID development.

In a study on a monozygotic twin pair discordant for CVID, the CVID-twin demonstrated impaired DNA demethylation in key B cell genes such as *PIK3CD*, *RPS6KB2*, *BCL2L1*, *TCF3*, *CORO1B*/*PTPRCAP*, *KCNN4* and *KCNC4*.[333] The B cell genes that were hypermethylated in the CVID-twin covered diverse functions of B cell biology.[333] Subsequent analysis of a larger CVID and healthy control cohort confirmed that CVID B cells had a reduced ability to demethylate these key genes during differentiation from naive to memory B cells.[333] DNA methylation is an epigenetic mechanism to control gene expression and can be influenced by environmental factors like smoking and infections.[333] The altered DNA methylation patterns in CVID B cells implicate a role for epigenetic and/or environmental factors in CVID pathogenesis.[333]

1.6.3.2 Disease-modifying variants associated with CVID

Some genetic variants seem to occur more frequently in the CVID population and may thus be a risk factor to disease development, although they do not suffice to establish a complete phenotype. Such disease-modifying variants have been reported in DNA repair genes (e.g. *MSH5*),[334] and in *FCGR2A*.[335] Furthermore, in individual CVID patients, certain variants may

influence the development of specific disease features like enteropathy or autoimmunity.[336-338]

1.6.3.3 Genome-wide association studies

A genome-wide association study (GWAS) from 2011 (363 CVID patients, 3031 controls) found an association with the HLA region, consistent with findings from prior linkage studies.[331,339] These researchers also identified a suggestive but non-significant association with a chromosome 8p locus containing *ADAM28*, *ADAM7*, *ADAMDEC1* and *STC1*.[331] In addition, CVID patients demonstrated an increased total copy number variation burden, suggesting a role for genomic instability in CVID pathogenesis.[331] Intra-exonic duplications in *ORC4L* were found to be most highly associated with CVID.[331] A GWAS in healthy Chinese males (n=3495) showed an association between serum IgG and the locus containing *TNFRSF13B* (encoding TACI).[340] However, the 2011 GWAS did not detect associations with the *TNFRSF13B* locus.[331] A GWAS from 2015 (778 CVID patients, 10999 controls) confirmed association with the HLA locus and also found associations with loci containing *CD21*, *ICOS*, *MSH5*, *TNFRSF13B*, and *CLEC16A*.[203] The *CLEC16A* locus had previously been associated with autoimmune disorders.[203] CLEC16A might provide a link between autoimmunity and B cell deficiency in CVID.[203]

1.7 Treatment and prognosis of CVID

The mainstay of treatment in CVID is lifelong immunoglobulin replacement therapy.[29,341] In addition, prophylactic antibiotics are often used to treat chronic or recurrent rhinosinusitis. In case of bronchiectasis, antiflogistic maintenance treatment with azithromycin can be administrated.[29,341] Autoimmune manifestations, lymphoproliferation and granulomatous disease are treated with corticosteroids and/or other immunosuppressive drugs.[29,341] HSCT is rarely performed because of the high morbidity and mortality risks, but can be an effective curative treatment in CVID patients with hematological malignancies or severe immune dysregulation.[33,342] Genetic testing can help to identify patients with monogenic disorders for which HSCT might be indicated, such as IL-21(R) deficiency, LRBA and CTLA-4 deficiencies, and APDS (PI3K hyperactivity) (see also section 1.6.2).[247,257,270,302] Identification of the molecular defect may also offer opportunities for targeted immunomodulatory treatments. Case studies have shown promise for the use of mTOR inhibitors in APDS, and the CTLA-4 mimetic abatacept in LRBA and CTLA-4 deficiencies (see also Figure 16).[252,271,274]

CVID patients suffering from disease-related complications have a significantly higher morbidity and mortality compared to patients with an infections-only phenotype.[53] In a 40-year follow-up study, the risk of death related to noninfectious complications was increased 11-fold.[50] Median overall survival in patients with noninfectious complications was approximately 35 years following diagnosis, whereas those without complications appeared to have a life expectancy similar to the general population.[50] Attempts to distinguish subgroups of patients at risk for complications by means of peripheral B or T cell immunophenotyping have failed (see sections 1.5.2.3 and 1.5.3.1).[58-60,122] Furthermore, genetic variants that uniformly predict disease-related complications have, so far, not been described.[36] Hence, the need for efficient prognostic biomarkers in CVID has yet to be fulfilled.[36]

References

1. Chaplin DD. Overview of the immune response. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S3-23.
2. Pieper K, Grimbacher B, Eibel H. B-cell biology and development. J Allergy Clin Immunol. 2013;131(4):959-71.
3. Schlissel M. Immunology: B-cell development in the gut. Nature. 2013;501(7465):42-3.
4. Monroe JG, Dorshkind K. Fate decisions regulating bone marrow and peripheral B lymphocyte development. Adv Immunol. 2007;95:1-50.
5. Bruton OC. Agammaglobulinemia. Pediatrics. 1952;9(6):722-8.
6. Fried AJ, Bonilla FA. Pathogenesis, diagnosis, and management of primary antibody deficiencies and infections. Clin Microbiol Rev. 2009;22(3):396-414.
7. Dullaers M, De Bruyne R, Ramadani F, Gould HJ, Gevaert P, Lambrecht BN. The who, where, and when of IgE in allergic airway disease. J Allergy Clin Immunol. 2012;129(3):635-45.
8. De Silva NS, Klein U. Dynamics of B cells in germinal centres. Nat Rev Immunol. 2015;15(3):137-48.
9. Litinskiy MB, Nardelli B, Hilbert DM, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol. 2002;3(9):822-9.
10. Rickert RC, Jellusova J, Miletic AV. Signaling by the tumor necrosis factor receptor superfamily in B-cell biology and disease. Immunol Rev. 2011;244(1):115-33.
11. Janda A, Bowen A, Greenspan NS, Casadevall A. Ig Constant Region Effects on Variable Region Structure and Function. Front Microbiol. 2016;7:22.
12. Picard C, Al-Herz W, Bousfiha A, et al. Primary Immunodeficiency Diseases: an Update on the Classification from the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015. J Clin Immunol. 2015;35(8):696-726.
13. Modell V, Quinn J, Orange J, Notarangelo LD, Modell F. Primary immunodeficiencies worldwide: an updated overview from the Jeffrey Modell Centers Global Network. Immunol Res. 2016;64(3):736-53.
14. Ochs HD, Hitzig WH. History of primary immunodeficiency diseases. Curr Opin Allergy Clin Immunol. 2012;12(6):577-87.
15. Meyts I, Bosch B, Bolze A, et al. Exome and genome sequencing for inborn errors of immunity. J Allergy Clin Immunol. 2016;138(4):957-69.
16. Milner JD, Holland SM. The cup runneth over: lessons from the ever-expanding pool of primary immunodeficiency diseases. Nat Rev Immunol. 2013;13(9):635-48.
17. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-23.
18. Christensen HO, Fandrup S. Inborn Immune Deficiency: A Handbook for Parents, Patients, Nurses and Doctors. 2nd ed. Denmark: Immun Defekt Foreningen; 2008.
19. Bousfiha AA, Jeddane L, Ailal F, et al. Primary immunodeficiency diseases worldwide: more common than generally thought. J Clin Immunol. 2013;33(1):1-7.
20. Bousfiha A, Jeddane L, Al-Herz W, et al. The 2015 IUIS Phenotypic Classification for Primary Immunodeficiencies. J Clin Immunol. 2015;35(8):727-38.
21. Lehman HK. Autoimmunity and Immune Dysregulation in Primary Immune Deficiency Disorders. Curr Allergy Asthma Rep. 2015;15(9):53.
22. Jonkman-Berk BM, van den Berg JM, Ten Berge IJ, et al. Primary immunodeficiencies in the Netherlands: national patient data demonstrate the increased risk of malignancy. Clin Immunol. 2015;156(2):154-62.
23. Mortaz E, Tabarsi P, Mansouri D, et al. Cancers Related to Immunodeficiencies: Update and Perspectives. Front Immunol. 2016;7:365.
24. Korf BR. Basic genetics. Prim Care. 2004;31(3):461-78, vii.
25. Schacherer J. Beyond the simplicity of Mendelian inheritance. C R Biol. 2016;339(7-8):284-8.
26. Cooper DN, Krawczak M, Polychronakos C, Tyler-Smith C, Kehrer-Sawatzki H. Where genotype is not predictive of phenotype: towards an understanding of the molecular basis of reduced penetrance in human inherited disease. Hum Genet. 2013;132(10):1077-130.

27. Bousfiha AA, Jeddane L, Ailal F, et al. A phenotypic approach for IUIS PID classification and diagnosis: guidelines for clinicians at the bedside. J Clin Immunol. 2013;33(6):1078-87.
28. Durandy A, Kracker S, Fischer A. Primary antibody deficiencies. Nat Rev Immunol. 2013;13(7):519-33.
29. Bonilla FA, Barlan I, Chapel H, et al. International Consensus Document (ICON): Common Variable Immunodeficiency Disorders. J Allergy Clin Immunol Pract. 2016;4(1):38-59.
30. Stiehm RE. The four most common pediatric immunodeficiencies. Adv Exp Med Biol. 2007;601:15-26.
31. Szczawinska-Poplonyk A, Tapolska-Jozwiak K, Samara H. The B-cell compartment in antibody-deficient infants and young children - developing common variable immunodeficiency or transient immune maturation? Ital J Pediatr. 2016;42(1):71.
32. Aghamohammadi A, Mohammadi J, Parvaneh N, et al. Progression of selective IgA deficiency to common variable immunodeficiency. Int Arch Allergy Immunol. 2008;147(2):87-92.
33. Chapel H, Cunningham-Rundles C. Update in understanding common variable immunodeficiency disorders (CVIDs) and the management of patients with these conditions. Br J Haematol. 2009;145(6):709-27.
34. Fudenberg H, Good RA, Goodman HC, et al. Primary immunodeficiencies. Report of a World Health Organization Committee. Pediatrics. 1971;47(5):927-46.
35. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol. 1999;93(3):190-7.
36. Chapel H. Common Variable Immunodeficiency Disorders (CVID) - Diagnoses of Exclusion, Especially Combined Immune Defects. J Allergy Clin Immunol Pract. 2016;4(6):1158-9.
37. Ameratunga R, Woon ST, Gillis D, Koopmans W, Steele R. New diagnostic criteria for common variable immune deficiency (CVID), which may assist with decisions to treat with intravenous or subcutaneous immunoglobulin. Clin Exp Immunol. 2013;174(2):203-11.
38. Ameratunga R, Brewerton M, Slade C, et al. Comparison of diagnostic criteria for common variable immunodeficiency disorder. Front Immunol. 2014;5:415.
39. Bertinchamp R, Gerard L, Boutboul D, Malphettes M, Fieschi C, Oksenhendler E. Exclusion of Patients with a Severe T-Cell Defect Improves the Definition of Common Variable Immunodeficiency. J Allergy Clin Immunol Pract. 2016;4(6):1147-57.
40. Ameratunga R, Gillis D, Steele R. Diagnostic criteria for common variable immunodeficiency disorders. J Allergy Clin Immunol Pract. 2016;4(5):1017-8.
41. Kutukculer N, Gulez N. The outcome of patients with unclassified hypogammaglobulinemia in early childhood. Pediatr Allergy Immunol. 2009;20(7):693-8.
42. van de Ven AA, van Montfrans JM. Clinical complications in pediatric CVID are not restricted to patients with severely reduced class-switched memory B cells. Pediatr Allergy Immunol. 2011;22(3):347-8.
43. van de Ven AA, van de Corput L, van Tilburg CM, et al. Lymphocyte characteristics in children with common variable immunodeficiency. Clin Immunol. 2010;135(1):63-71.
44. Carvalho Neves Forte W, Ferreira De Carvalho Junior F, Damaceno N, Vidal Perez F, Gonzales Lopes C, Mastroti RA. Evolution of IgA deficiency to IgG subclass deficiency and common variable immunodeficiency. Allergol Immunopathol (Madr). 2000;28(1):18-20.
45. Bogaert DJ, De Bruyne M, Debacker V, et al. The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. Haematologica. 2017;102(1):192-202.
46. Bergbreiter A, Salzer U. Common variable immunodeficiency: a multifaceted and puzzling disorder. Expert Rev Clin Immunol. 2009;5(2):167-80.
47. Resnick ES, Cunningham-Rundles C. The many faces of the clinical picture of common variable immune deficiency. Curr Opin Allergy Clin Immunol. 2012;12(6):595-601.
48. Gathmann B, Mahlaoui N, Gerard L, et al. Clinical picture and treatment of 2212 patients with common variable immunodeficiency. J Allergy Clin Immunol. 2014;134(1):116-26.
49. Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. Clin Immunol. 1999;92(1):34-48.

50. Resnick ES, Moshier EL, Godbold JH, Cunningham-Rundles C. Morbidity and mortality in common variable immune deficiency over 4 decades. Blood. 2012;119(7):1650-7.
51. Chapel H, Lucas M, Patel S, et al. Confirmation and improvement of criteria for clinical phenotyping in common variable immunodeficiency disorders in replicate cohorts. J Allergy Clin Immunol. 2012;130(5):1197-1198.e9.
52. Patuzzo G, Barbieri A, Tinazzi E, et al. Autoimmunity and infection in common variable immunodeficiency (CVID). Autoimmun Rev. 2016;15(9):877-82.
53. Chapel H, Lucas M, Lee M, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood. 2008;112(2):277-86.
54. Malphettes M, Gerard L, Carmagnat M, et al. Late-onset combined immune deficiency: a subset of common variable immunodeficiency with severe T cell defect. Clin Infect Dis. 2009;49(9):1329-38.
55. Boileau J, Mouillot G, Gerard L, et al. Autoimmunity in common variable immunodeficiency: correlation with lymphocyte phenotype in the French DEFI study. J Autoimmun. 2011;36(1):25-32.
56. Gangemi S, Allegra A, Musolino C. Lymphoproliferative disease and cancer among patients with common variable immunodeficiency. Leuk Res. 2015;39(4):389-96.
57. Warnatz K, Voll RE. Pathogenesis of autoimmunity in common variable immunodeficiency. Front Immunol. 2012;3:210.
58. Warnatz K, Denz A, Drager R, et al. Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood. 2002;99(5):1544-51.
59. Piqueras B, Lavenu-Bombled C, Galicier L, et al. Common variable immunodeficiency patient classification based on impaired B cell memory differentiation correlates with clinical aspects. J Clin Immunol. 2003;23(5):385-400.
60. Wehr C, Kivioja T, Schmitt C, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood. 2008;111(1):77-85.
61. Ochtrop ML, Goldacker S, May AM, et al. T and B lymphocyte abnormalities in bone marrow biopsies of common variable immunodeficiency. Blood. 2011;118(2):309-18.
62. Isgro A, Marziali M, Mezzaroma I, et al. Bone marrow clonogenic capability, cytokine production, and thymic output in patients with common variable immunodeficiency. J Immunol. 2005;174(8):5074-81.
63. Eisenstein EM, Strober W. Evidence for a generalized signaling abnormality in B cells from patients with common variable immunodeficiency. Adv Exp Med Biol. 1995;371B:699-704.
64. Denz A, Eibel H, Illges H, Kienzle G, Schlesier M, Peter HH. Impaired up-regulation of CD86 in B cells of "type A" common variable immunodeficiency patients. Eur J Immunol. 2000;30(4):1069-77.
65. Groth C, Drager R, Warnatz K, et al. Impaired up-regulation of CD70 and CD86 in naive (CD27-) B cells from patients with common variable immunodeficiency (CVID). Clin Exp Immunol. 2002;129(1):133-9.
66. Foerster C, Voelxen N, Rakhmanov M, et al. B cell receptor-mediated calcium signaling is impaired in B lymphocytes of type Ia patients with common variable immunodeficiency. J Immunol. 2010;184(12):7305-13.
67. van de Ven AA, Compeer EB, Bloem AC, et al. Defective calcium signaling and disrupted CD20-B-cell receptor dissociation in patients with common variable immunodeficiency disorders. J Allergy Clin Immunol. 2012;129(3):755-761.e7.
68. Barbosa RR, Silva SL, Silva SP, et al. Reduced BAFF-R and increased TACI expression in common variable immunodeficiency. J Clin Immunol. 2014;34(5):573-83.
69. Guo BC, Saxon A. B cell lines from a subset of patients with common variable immunodeficiency undergo enhanced apoptosis associated with an increased display of CD95 (Apo-1/fas), diminished CD38 expression, and decreased IgG and IgA production. Cell Immunol. 1995;166(1):83-92.
70. Savasan S, Warrier I, Buck S, Kaplan J, Ravindranath Y. Increased lymphocyte Fas expression and high incidence of common variable immunodeficiency disorder in childhood Evans' syndrome. Clin Immunol. 2007;125(3):224-9.

71. Piatosa B, Pac M, Siewiera K, et al. Common variable immune deficiency in children--clinical characteristics varies depending on defect in peripheral B cell maturation. J Clin Immunol. 2013;33(4):731-41.
72. Weston SA, Prasad ML, Mullighan CG, Chapel H, Benson EM. Assessment of male CVID patients for mutations in the Btk gene: how many have been misdiagnosed? Clin Exp Immunol. 2001;124(3):465-9.
73. Kanegane H, Tsukada S, Iwata T, et al. Detection of Bruton's tyrosine kinase mutations in hypogammaglobulinaemic males registered as common variable immunodeficiency (CVID) in the Japanese Immunodeficiency Registry. Clin Exp Immunol. 2000;120(3):512-7.
74. Serana F, Airo P, Chiarini M, et al. Thymic and bone marrow output in patients with common variable immunodeficiency. J Clin Immunol. 2011;31(4):540-9.
75. Bryant A, Calver NC, Toubi E, Webster AD, Farrant J. Classification of patients with common variable immunodeficiency by B cell secretion of IgM and IgG in response to anti-IgM and interleukin-2. Clin Immunol Immunopathol. 1990;56(2):239-48.
76. Borte S, Pan-Hammarstrom Q, Liu C, et al. Interleukin-21 restores immunoglobulin production ex vivo in patients with common variable immunodeficiency and selective IgA deficiency. Blood. 2009;114(19):4089-98.
77. Driessen GJ, van Zelm MC, van Hagen PM, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood. 2011;118(26):6814-23.
78. Brouet JC, Chedeville A, Fermand JP, Royer B. Study of the B cell memory compartment in common variable immunodeficiency. Eur J Immunol. 2000;30(9):2516-20.
79. Artus U, Herbst EW, Rump JA, Peter HH. Defects in the immunoglobulin producing cells in bone marrow of patients with variable immunodeficiency syndrome. Immun Infekt. 1995;23(2):69-71.
80. Herbst EW, Armbruster M, Rump JA, Buscher HP, Peter HH. Intestinal B cell defects in common variable immunodeficiency. Clin Exp Immunol. 1994;95(2):215-21.
81. Agarwal S, Smereka P, Harpaz N, Cunningham-Rundles C, Mayer L. Characterization of immunologic defects in patients with common variable immunodeficiency (CVID) with intestinal disease. Inflamm Bowel Dis. 2011;17(1):251-9.
82. Sander CA, Medeiros LJ, Weiss LM, Yano T, Sneller MC, Jaffe ES. Lymphoproliferative lesions in patients with common variable immunodeficiency syndrome. Am J Surg Pathol. 1992;16(12):1170-82.
83. Taubenheim N, von Hornung M, Durandy A, et al. Defined blocks in terminal plasma cell differentiation of common variable immunodeficiency patients. J Immunol. 2005;175(8):5498-503.
84. Unger S, Seidl M, Schmitt-Graeff A, et al. Ill-Defined Germinal Centers and Severely Reduced Plasma Cells are Histological Hallmarks of Lymphadenopathy in Patients with Common Variable Immunodeficiency. J Clin Immunol. 2014;34(6):615-26.
85. Bonhomme D, Hammarstrom L, Webster D, et al. Impaired antibody affinity maturation process characterizes a subset of patients with common variable immunodeficiency. J Immunol. 2000;165(8):4725-30.
86. Andersen P, Permin H, Andersen V, et al. Deficiency of somatic hypermutation of the antibody light chain is associated with increased frequency of severe respiratory tract infection in common variable immunodeficiency. Blood. 2005;105(2):511-7.
87. Schejbel L, Marquart H, Andersen V, et al. Deficiency of somatic hypermutation of immunoglobulin G transcripts is a better predictor of severe respiratory tract infections than lack of memory B cells in common variable immunodeficiency. J Clin Immunol. 2005;25(4):392-403.
88. Levy Y, Gupta N, Le Deist F, et al. Defect in IgV gene somatic hypermutation in common variable immuno-deficiency syndrome. Proc Natl Acad Sci U S A. 1998;95(22):13135-40.
89. Clemente A, Pons J, Lanio N, Matamoros N, Ferrer JM. CD27+ B cells from a subgroup of common variable immunodeficiency patients are less sensitive to apoptosis rescue regardless of interleukin-21 signalling. Clin Exp Immunol. 2013;174(1):97-108.
90. Weller S, Braun MC, Tan BK, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood. 2004;104(12):3647-54.

91. Carsetti R, Rosado MM, Donnanno S, et al. The loss of IgM memory B cells correlates with clinical disease in common variable immunodeficiency. J Allergy Clin Immunol. 2005;115(2):412-7.
92. Rakhmanov M, Keller B, Gutenberger S, et al. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. Proc Natl Acad Sci U S A. 2009;106(32):13451-6.
93. Isnardi I, Ng YS, Menard L, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. Blood. 2010;115(24):5026-36.
94. Thorarinsdottir K, Camponeschi A, Gjertsson I, Martensson IL. CD21 -/low B cells: A Snapshot of a Unique B Cell Subset in Health and Disease. Scand J Immunol. 2015;82(3):254-61.
95. Vlkova M, Fronkova E, Kanderova V, et al. Characterization of lymphocyte subsets in patients with common variable immunodeficiency reveals subsets of naive human B cells marked by CD24 expression. J Immunol. 2010;185(11):6431-8.
96. Wehr C, Eibel H, Masilamani M, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. Clin Immunol. 2004;113(2):161-71.
97. Terrier B, Joly F, Vazquez T, et al. Expansion of functionally anergic CD21-/low marginal zone-like B cell clones in hepatitis C virus infection-related autoimmunity. J Immunol. 2011;187(12):6550-63.
98. Moir S, Fauci AS. Pathogenic mechanisms of B-lymphocyte dysfunction in HIV disease. The J Allergy Clin Immunol. 2008;122(1):12-9; quiz 20-1.
99. Moir S, Ho J, Malaspina A, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. J Exp Med. 2008;205(8):1797-805.
100. Warnatz K, Schlesier M. Flowcytometric phenotyping of common variable immunodeficiency. Cytometry B Clin Cytom. 2008;74(5):261-71.
101. Warnatz K, Wehr C, Drager R, et al. Expansion of CD19(hi)CD21(lo/neg) B cells in common variable immunodeficiency (CVID) patients with autoimmune cytopenia. Immunobiology. 2002;206(5):502-13.
102. Ballegaard V, Permin H, Katzenstein TL, Marquart HV, Schejbel L. Long-term follow-up on affinity maturation and memory B-cell generation in patients with common variable immunodeficiency. J Clin Immunol. 2013;33(6):1067-77.
103. Kutukculer N, Gulez N, Karaca NE, Aksu G, Berdeli A. Three different classifications, B lymphocyte subpopulations, TNFRSF13B (TACI), TNFRSF13C (BAFF-R), TNFSF13 (APRIL) gene mutations, CTLA-4 and ICOS gene polymorphisms in Turkish patients with common variable immunodeficiency. J Clin Immunol. 2012;32(6):1165-79.
104. Koopmans W, Woon ST, Zeng IS, et al. Variability of memory B cell markers in a cohort of common variable immune deficiency patients over 6 months. Scand J Immunol. 2013;77(6):470-5.
105. Al Kindi M, Mundy J, Sullivan T, et al. Utility of peripheral blood B cell subsets analysis in common variable immunodeficiency. Clin Exp Immunol. 2012;167(2):275-81.
106. Driessen GJ, Dalm VA, van Hagen PM, et al. Common variable immunodeficiency and idiopathic primary hypogammaglobulinemia: two different conditions within the same disease spectrum. Haematologica. 2013;98(10):1617-23.
107. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol. 2014;32:659-702.
108. Payne D, Drinkwater S, Baretto R, Duddridge M, Browning MJ. Expression of chemokine receptors CXCR4, CXCR5 and CCR7 on B and T lymphocytes from patients with primary antibody deficiency. Clin Exp Immunol. 2009;156(2):254-62.
109. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637-50.
110. Crampton SP, Voynova E, Bolland S. Innate pathways to B-cell activation and tolerance. Ann N Y Acad Sci. 2010;1183:58-68.
111. Bernasconi NL, Onai N, Lanzavecchia A. A role for Toll-like receptors in acquired immunity: up-regulation of TLR9 by BCR triggering in naive B cells and constitutive expression in memory B cells. Blood. 2003;101(11):4500-4.

112. Escobar D, Pons J, Clemente A, et al. Defective B cell response to TLR9 ligand (CpG-ODN), Streptococcus pneumoniae and Haemophilus influenzae extracts in common variable immunodeficiency patients. Cell Immunol. 2010;262(2):105-11.
113. Cunningham-Rundles C, Radigan L, Knight AK, Zhang L, Bauer L, Nakazawa A. TLR9 activation is defective in common variable immune deficiency. J Immunol. 2006;176(3):1978-87.
114. Clemente A, Pons J, Matamoros N, Iglesias J, Ferrer JM. B cells from common variable immunodeficiency patients fail to differentiate to antibody secreting cells in response to TLR9 ligand (CpG-ODN) or anti-CD40+IL21. Cell Immunol. 2011;268(1):9-15.
115. Yu JE, Knight AK, Radigan L, et al. Toll-like receptor 7 and 9 defects in common variable immunodeficiency. J Allergy Clin Immunol. 2009;124(2):349-56, 356.e1-3.
116. Lougaris V, Baronio M, Vitali M, et al. B cell responses to CpG correlate with CXCL16 expression levels in common variable immunodeficiency. ScientificWorldJournal. 2012;2012:960219.
117. Tanir S, Karkucak M, Yakut T, Kilic SS. Toll-like receptor-9 gene polymorphism in common variable immunodeficiency. J Investig Allergol Clin Immunol. 2010;20(3):267-8.
118. Yu JE, Zhang L, Radigan L, Sanchez-Ramon S, Cunningham-Rundles C. TLR-mediated B cell defects and IFN-alpha in common variable immunodeficiency. J Clin Immunol. 2012;32(1):50-60.
119. Marron TU, Yu JE, Cunningham-Rundles C. Toll-like receptor function in primary B cell defects. Front Biosci (Elite Ed). 2012;4:1853-63.
120. Wong GK, Huissoon AP. T-cell abnormalities in common variable immunodeficiency: the hidden defect. J Clin Pathol. 2016;69(8):672-6.
121. Mouillot G, Carmagnat M, Gerard L, et al. B-cell and T-cell phenotypes in CVID patients correlate with the clinical phenotype of the disease. J Clin Immunol. 2010;30(5):746-55.
122. Giovannetti A, Pierdominici M, Mazzetta F, et al. Unravelling the complexity of T cell abnormalities in common variable immunodeficiency. J Immunol. 2007;178(6):3932-43.
123. Oraei M, Aghamohammadi A, Rezaei N, et al. Naive CD4+ T cells and recent thymic emigrants in common variable immunodeficiency. J Investig Allergol Clin Immunol. 2012;22(3):160-7.
124. Bateman EA, Ayers L, Sadler R, et al. T cell phenotypes in patients with common variable immunodeficiency disorders: associations with clinical phenotypes in comparison with other groups with recurrent infections. Clin Exp Immunol. 2012;170(2):202-11.
125. Guazzi V, Aiuti F, Mezzaroma I, et al. Assessment of thymic output in common variable immunodeficiency patients by evaluation of T cell receptor excision circles. Clin Exp Immunol. 2002;129(2):346-53.
126. De Vera MJ, Al-Harthi L, Gewurz AT. Assessing thymopoiesis in patients with common variable immunodeficiency as measured by T-cell receptor excision circles. Ann Allergy Asthma Immunol. 2004;93(5):478-84.
127. Ramesh M, Hamm D, Simchoni N, Cunningham-Rundles C. Clonal and constricted T cell repertoire in Common Variable Immune Deficiency. Clin Immunol. 2015. pii: S1521-6616(15)00004-2.
128. Di Renzo M, Serrano D, Zhou Z, George I, Becker K, Cunningham-Rundles C. Enhanced T cell apoptosis in common variable immunodeficiency: negative role of the fas/fasligand system and of the Bcl-2 family proteins and possible role of TNF-RS. Clin Exp Immunol. 2001;125(1):117-22.
129. Carbone J, Sarmiento E, Micheloud D, Rodriguez-Molina J, Fernandez-Cruz E. Elevated levels of activated CD4 T cells in common variable immunodeficiency: association with clinical findings. Allergol Immunopathol (Madr). 2006;34(4):131-5.
130. Fevang B, Yndestad A, Sandberg WJ, et al. Low numbers of regulatory T cells in common variable immunodeficiency: association with chronic inflammation in vivo. Clin Exp Immunol. 2007;147(3):521-5.
131. Genre J, Errante PR, Kokron CM, Toledo-Barros M, Camara NO, Rizzo LV. Reduced frequency of CD4(+)CD25(HIGH)FOXP3(+) cells and diminished FOXP3 expression in patients with Common Variable Immunodeficiency: a link to autoimmunity? Clin Immunol. 2009;132(2):215-21.
132. Arumugakani G, Wood PM, Carter CR. Frequency of Treg cells is reduced in CVID patients with autoimmunity and splenomegaly and is associated with expanded CD21lo B lymphocytes. J Clin Immunol. 2010;30(2):292-300.
133. Horn J, Manguiat A, Berglund LJ, et al. Decrease in phenotypic regulatory T cells in subsets of patients with common variable immunodeficiency. Clin Exp Immunol. 2009;156(3):446-54.

134. Yu GP, Chiang D, Song SJ, et al. Regulatory T cell dysfunction in subjects with common variable immunodeficiency complicated by autoimmune disease. Clin Immunol. 2009;131(2):240-53.
135. Carter CR, Aravind G, Smalle NL, Cole JY, Savic S, Wood PM. CVID patients with autoimmunity have elevated T cell expression of granzyme B and HLA-DR and reduced levels of Treg cells. J Clin Pathol. 2013;66(2):146-50.
136. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nat Rev Immunol. 2008;8(7):523-32.
137. Arandi N, Mirshafiey A, Abolhassani H, et al. Frequency and expression of inhibitory markers of CD4(+) CD25(+) FOXP3(+) regulatory T cells in patients with common variable immunodeficiency. Scand J Immunol. 2013;77(5):405-12.
138. Arandi N, Mirshafiey A, Jeddi-Tehrani M, et al. Evaluation of CD4+CD25+FOXP3+ regulatory T cells function in patients with common variable immunodeficiency. Cell Immunol. 2013;281(2):129-33.
139. Bossaller L, Burger J, Draeger R, et al. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. J Immunol. 2006;177(7):4927-32.
140. Romberg ND, Hsu I, Price CC, Cunningham-Rundles C, Meffre E. Expansion Of Circulating T Follicular Helper Cells In CVID Patients With Autoimmune Cytopenias. J Allergy Clin Immunol. 2014;133(2 Suppl):AB162.
141. Pandolfi F, Trentin L, San Martin JE, Wong JT, Kurnick JT, Moscicki RA. T cell heterogeneity in patients with common variable immunodeficiency as assessed by abnormalities of T cell subpopulations and T cell receptor gene analysis. Clin Exp Immunol. 1992;89(2):198-203.
142. Viallard JF, Ruiz C, Guillet M, Pellegrin JL, Moreau JF. Perturbations of the CD8(+) T-cell repertoire in CVID patients with complications. Results Immunol. 2013;3:122-8.
143. Viallard JF, Blanco P, Andre M, et al. CD8+HLA-DR+ T lymphocytes are increased in common variable immunodeficiency patients with impaired memory B-cell differentiation. Clin Immunol. 2006;119(1):51-8.
144. Wright JJ, Wagner DK, Blaese RM, Hagengruber C, Waldmann TA, Fleisher TA. Characterization of common variable immunodeficiency: identification of a subset of patients with distinctive immunophenotypic and clinical features. Blood. 1990;76(10):2046-51.
145. Jaffe JS, Eisenstein E, Sneller MC, Strober W. T-cell abnormalities in common variable immunodeficiency. Pediatr Res. 1993;33(1 Suppl):S24-7; discussion S27-8.
146. Serrano D, Becker K, Cunningham-Rundles C, Mayer L. Characterization of the T cell receptor repertoire in patients with common variable immunodeficiency: oligoclonal expansion of CD8(+) T cells. Clin Immunol. 2000;97(3):248-58.
147. Vultaggio A, Matucci A, D'Elios MM, et al. Multiorgan infiltration by CD8+ T cells and 1p;16p translocation in a patient with hypogammaglobulinemia and a reduced number of B cells. Int Arch Allergy Immunol. 2012;158(2):206-10.
148. Jaffe JS, Strober W, Sneller MC. Functional abnormalities of CD8+ T cells define a unique subset of patients with common variable immunodeficiency. Blood. 1993;82(1):192-201.
149. Holm AM, Sivertsen EA, Tunheim SH, et al. Gene expression analysis of peripheral T cells in a subgroup of common variable immunodeficiency shows predominance of CCR7(-) effector-memory T cells. Clin Exp Immunol. 2004;138(2):278-89.
150. Holm AM, Aukrust P, Damas JK, Muller F, Halvorsen B, Froland SS. Abnormal interleukin-7 function in common variable immunodeficiency. Blood. 2005;105(7):2887-90.
151. Picat M-Q, Thiebaut R, Lifermann F, et al. T-cell activation discriminates subclasses of symptomatic primary humoral immunodeficiency diseases in adults. BMC Immunol. 2014;15:13.
152. Marashi SM, Raeiszadeh M, Workman S, et al. Inflammation in common variable immunodeficiency is associated with a distinct CD8(+) response to cytomegalovirus. J Allergy Clin Immunol. 2011;127(6):1385-93.e4.
153. Marashi SM, Raeiszadeh M, Enright V, et al. Influence of cytomegalovirus infection on immune cell phenotypes in patients with common variable immunodeficiency. J Allergy Clin Immunol. 2012;129(5):1349-1356.e3.
154. Cunningham-Rundles S, Cunningham-Rundles C, Siegal FP, et al. Defective cellular immune response in vitro in common variable immunodeficiency. J Clin Immunol. 1981;1(1):65-72.

155. Fischer MB, Hauber I, Eggenbauer H, et al. A defect in the early phase of T-cell receptor-mediated T-cell activation in patients with common variable immunodeficiency. Blood. 1994;84(12):4234-41.
156. Aukrust P, Aandahl EM, Skalhegg BS, et al. Increased activation of protein kinase A type I contributes to the T cell deficiency in common variable immunodeficiency. J Immunol. 1999;162(2):1178-85.
157. Varzaneh FN, Keller B, Unger S, Aghamohammadi A, Warnatz K, Rezaei N. Cytokines in common variable immunodeficiency as signs of immune dysregulation and potential therapeutic targets - a review of the current knowledge. J Clin Immunol. 2014;34(5):524-43.
158. Rezaei N, Aghamohammadi A, Mahmoudi M, et al. Association of IL-4 and IL-10 gene promoter polymorphisms with common variable immunodeficiency. Immunobiology. 2010;215(1):81-7.
159. Rezaei N, Aghamohammadi A, Shakiba Y, et al. Cytokine gene polymorphisms in common variable immunodeficiency. Int Arch Allergy Immunol. 2009;150(1):1-7.
160. Rezaei N, Amirzargar AA, Shakiba Y, Mahmoudi M, Moradi B, Aghamohammadi A. Proinflammatory cytokine gene single nucleotide polymorphisms in common variable immunodeficiency. Clin Exp Immunol. 2009;155(1):21-7.
161. Aukrust P, Froland SS, Liabakk NB, et al. Release of cytokines, soluble cytokine receptors, and interleukin-1 receptor antagonist after intravenous immunoglobulin administration in vivo. Blood. 1994;84(7):2136-43.
162. Ibanez C, Sune P, Fierro A, et al. Modulating effects of intravenous immunoglobulins on serum cytokine levels in patients with primary hypogammaglobulinemia. BioDrugs. 2005;19(1):59-65.
163. Cunningham-Rundles C, Bodian C, Ochs HD, Martin S, Reiter-Wong M, Zhuo Z. Long-term low-dose IL-2 enhances immune function in common variable immunodeficiency. Clin Immunol. 2001;100(2):181-90.
164. Cunningham-Rundles C, Mayer L, Sapira E, Mendelsohn L. Restoration of immunoglobulin secretion in vitro in common variable immunodeficiency by in vivo treatment with polyethylene glycol-conjugated human recombinant interleukin-2. Clin Immunol Immunopathol. 1992;64(1):46-56.
165. Rump JA, Jahreis A, Schlesier M, Stecher S, Peter HH. A double-blind, placebo-controlled, crossover therapy study with natural human IL-2 (nhuIL-2) in combination with regular intravenous gammaglobulin (IVIG) infusions in 10 patients with common variable immunodeficiency (CVID). Clin Exp Immunol. 1997;110(2):167-73.
166. Cunningham-Rundles C, Kazbay K, Hassett J, Zhou Z, Mayer L. Brief report: enhanced humoral immunity in common variable immunodeficiency after long-term treatment with polyethylene glycol-conjugated interleukin-2. N Engl J Med. 1994;331(14):918-21.
167. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu Rev Immunol. 2009;27:669-92.
168. Aukrust P, Muller F, Froland SS. Enhanced generation of reactive oxygen species in monocytes from patients with common variable immunodeficiency. Clin Exp Immunol. 1994;97(2):232-8.
169. Aukrust P, Lien E, Kristoffersen AK, et al. Persistent activation of the tumor necrosis factor system in a subgroup of patients with common variable immunodeficiency--possible immunologic and clinical consequences. Blood. 1996;87(2):674-81.
170. Aukrust P, Froland SS, Muller F. Raised serum neopterin levels in patients with primary hypogammaglobulinaemia; correlation to other immunological parameters and to clinical and histological features. Clin Exp Immunol. 1992;89(2):211-6.
171. Barbosa RR, Silva SP, Silva SL, et al. Monocyte activation is a feature of common variable immunodeficiency irrespective of plasma lipopolysaccharide levels. Clin Exp Immunol. 2012;169(3):263-72.
172. Cambronero R, Sewell WA, North ME, Webster AD, Farrant J. Up-regulation of IL-12 in monocytes: a fundamental defect in common variable immunodeficiency. J Immunol. 2000;164(1):488-94.
173. Hong R, Agrawal S, Gollapudi S, Gupta S. Impaired pneumovax-23-induced monocyte-derived cytokine production in patients with common variable immunodeficiency. J Clin Immunol. 2010;30(3):435-41.

174. Amoras AL, Kanegane H, Miyawaki T, Vilela MM. Defective Fc-, CR1- and CR3-mediated monocyte phagocytosis and chemotaxis in common variable immunodeficiency and X-linked agammaglobulinemia patients. J Investig Allergol Clin Immunol. 2003;13(3):181-8.
175. Sichien D, Lambrecht BN, Guilliams M, Scott CL. Development of conventional dendritic cells: from common bone marrow progenitors to multiple subsets in peripheral tissues. Mucosal Immunol. 2017 Feb 15. [Epub ahead of print].
176. Poeck H, Wagner M, Battiany J, et al. Plasmacytoid dendritic cells, antigen, and CpG-C license human B cells for plasma cell differentiation and immunoglobulin production in the absence of T-cell help. Blood. 2004;103(8):3058-64.
177. Martinez-Pomar N, Raga S, Ferrer J, et al. Elevated serum interleukin (IL)-12p40 levels in common variable immunodeficiency disease and decreased peripheral blood dendritic cells: analysis of IL-12p40 and interferon-gamma gene. Clin Exp Immunol. 2006;144(2):233-8.
178. Viallard JF, Camou F, Andre M, et al. Altered dendritic cell distribution in patients with common variable immunodeficiency. Arthritis Res Ther. 2005;7(5):R1052-5.
179. Yong PF, Workman S, Wahid F, Exley A, Webster AD, Ibrahim MA. Selective deficits in blood dendritic cell subsets in common variable immunodeficiency and X-linked agammaglobulinaemia but not specific polysaccharide antibody deficiency. Clin Immunol. 2008;127(1):34-42.
180. Taraldsrud E, Fevang B, Aukrust P, et al. Common variable immunodeficiency revisited: normal generation of naturally occurring dendritic cells that respond to Toll-like receptors 7 and 9. Clin Exp Immunol. 2014;175(3):439-48.
181. Trujillo CM, Muskus C, Arango J, Patino PJ, Montoya CJ. Quantitative and functional evaluation of innate immune responses in patients with common variable immunodeficiency. J Investig Allergol Clin Immunol. 2011;21(3):207-15.
182. Bayry J, Lacroix-Desmazes S, Kazatchkine MD, et al. Common variable immunodeficiency is associated with defective functions of dendritic cells. Blood. 2004;104(8):2441-3.
183. Cunningham-Rundles C, Radigan L. Deficient IL-12 and dendritic cell function in common variable immune deficiency. Clin Immunol. 2005;115(2):147-53.
184. Scott-Taylor TH, Green MR, Eren E, Webster AD. Monocyte derived dendritic cell responses in common variable immunodeficiency. Clin Exp Immunol. 2004;138(3):484-90.
185. Lam VC, Lanier LL. NK cells in host responses to viral infections. Curr Opin Immunol. 2016;44:43-51.
186. Aspalter RM, Sewell WA, Dolman K, Farrant J, Webster AD. Deficiency in circulating natural killer (NK) cell subsets in common variable immunodeficiency and X-linked agammaglobulinaemia. Clin Exp Immunol. 2000;121(3):506-14.
187. Kutukculer N, Azarsiz E, Karaca NE, Ulusoy E, Koturoglu G, Aksu G. A Clinical and Laboratory Approach to the Evaluation of Innate Immunity in Pediatric CVID Patients. Front Immunol. 2015;6:145.
188. Bayry J, Hermine O, Webster DA, Levy Y, Kaveri SV. Common variable immunodeficiency: the immune system in chaos. Trends Mol Med. 2005;11(8):370-6.
189. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. Annu Rev Immunol. 2005;23:877-900.
190. Lang GA, Devera TS, Lang ML. Requirement for CD1d expression by B cells to stimulate NKT cell-enhanced antibody production. Blood. 2008;111(4):2158-62.
191. Carvalho KI, Melo KM, Bruno FR, et al. Skewed distribution of circulating activated natural killer T (NKT) cells in patients with common variable immunodeficiency disorders (CVID). PLoS One. 2010;5(9). pii: e12652.
192. Fulcher DA, Avery DT, Fewings NL, et al. Invariant natural killer (iNK) T cell deficiency in patients with common variable immunodeficiency. Clin Exp Immunol. 2009;157(3):365-9.
193. Gao Y, Workman S, Gadola S, Elliott T, Grimbacher B, Williams AP. Common variable immunodeficiency is associated with a functional deficiency of invariant natural killer T cells. J Allergy Clin Immunol. 2014;133(5):1420-8, 1428.e1.
194. Paquin-Proulx D, Santos BA, Carvalho KI, et al. IVIg immune reconstitution treatment alleviates the state of persistent immune activation and suppressed CD4 T cell counts in CVID. PLoS One. 2013;8(10):e75199.

195. Walker JA, Barlow JL, McKenzie AN. Innate lymphoid cells--how did we miss them? Nat Rev Immunol. 2013;13(2):75-87.
196. Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. Nat Immunol. 2016;17(7):765-74.
197. Cols M, Rahman A, Maglione PJ, et al. Expansion of inflammatory innate lymphoid cells in patients with common variable immune deficiency. J Allergy Clin Immunol. 2016;137(4):1206-15.e1-6.
198. van Schouwenburg PA, Davenport EE, Kienzler AK, et al. Application of whole genome and RNA sequencing to investigate the genomic landscape of common variable immunodeficiency disorders. Clin Immunol. 2015;160(2):301-14.
199. Grimbacher B, Hutloff A, Schlesier M, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol. 2003;4(3):261-8.
200. Castigli E, Wilson SA, Garibyan L, et al. TACI is mutant in common variable immunodeficiency and IgA deficiency. Nat Genet. 2005;37(8):829-34.
201. Salzer U, Chapel HM, Webster AD, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. Nat Genet. 2005;37(8):820-8.
202. Warnatz K, Salzer U, Rizzi M, et al. B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. Proc Natl Acad Sci U S A. 2009;106(33):13945-50.
203. Li J, Jorgensen SF, Maggadottir SM, et al. Association of CLEC16A with human common variable immunodeficiency disorder and role in murine B cells. Nat Commun. 2015;6:6804.
204. Salzer U, Maul-Pavicic A, Cunningham-Rundles C, et al. ICOS deficiency in patients with common variable immunodeficiency. Clin Immunol. 2004;113(3):234-40.
205. Takahashi N, Matsumoto K, Saito H, et al. Impaired CD4 and CD8 effector function and decreased memory T cell populations in ICOS-deficient patients. J Immunol. 2009;182(9):5515-27.
206. Chou J, Massaad MJ, Cangemi B, et al. A novel mutation in ICOS presenting as hypogammaglobulinemia with susceptibility to opportunistic pathogens. J Allergy Clin Immunol. 2015;136(3):794-797.e1.
207. Robertson N, Engelhardt KR, Morgan NV, et al. Astute Clinician Report: A Novel 10 bp Frameshift Deletion in Exon 2 of ICOS Causes a Combined Immunodeficiency Associated with an Enteritis and Hepatitis. J Clin Immunol. 2015;35(7):598-603.
208. Warnatz K, Bossaller L, Salzer U, et al. Human ICOS deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. Blood. 2006;107(8):3045-52.
209. Zhang Y, Li J, Zhang YM, Zhang XM, Tao J. Effect of TACI signaling on humoral immunity and autoimmune diseases. J Immunol Res. 2015;2015:247426.
210. Castigli E, Wilson S, Garibyan L, et al. Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. Nat Genet. 2007;39(4):430-1.
211. Pan-Hammarstrom Q, Salzer U, Du L, et al. Reexamining the role of TACI coding variants in common variable immunodeficiency and selective IgA deficiency. Nat Genet. 2007;39(4):429-30.
212. Salzer U, Bacchelli C, Buckridge S, et al. Relevance of biallelic versus monoallelic TNFRSF13B mutations in distinguishing disease-causing from risk-increasing TNFRSF13B variants in antibody deficiency syndromes. Blood. 2009;113(9):1967-76.
213. Martinez-Gallo M, Radigan L, Almejun MB, Martinez-Pomar N, Matamoros N, Cunningham-Rundles C. TACI mutations and impaired B-cell function in subjects with CVID and healthy heterozygotes. J Allergy Clin Immunol. 2013;131(2):468-76.
214. Pieper K, Rizzi M, Speletas M, et al. A common single nucleotide polymorphism impairs B-cell activating factor receptor's multimerization, contributing to common variable immunodeficiency. J Allergy Clin Immunol. 2014;133(4):1222-5.
215. Lougaris V, Gallizzi R, Vitali M, et al. A novel compound heterozygous TACI mutation in an autosomal recessive common variable immunodeficiency (CVID) family. Hum Immunol. 2012;73(8):836-9.
216. Martinez-Pomar N, Detkova D, Arostegui JI, et al. Role of TNFRSF13B variants in patients with common variable immunodeficiency. Blood. 2009;114(13):2846-8.

217. Mohammadi J, Liu C, Aghamohammadi A, et al. Novel mutations in TACI (TNFRSF13B) causing common variable immunodeficiency. J Clin Immunol. 2009;29(6):777-85.
218. Speletas M, Mamara A, Papadopoulou-Alataki E, et al. TNFRSF13B/TACI alterations in Greek patients with antibody deficiencies. J Clin Immunol. 2011;31(4):550-9.
219. Almejun MB, Cols M, Zelazko M, et al. Naturally occurring mutation affecting the MyD88-binding site of TNFRSF13B impairs triggering of class switch recombination. Eur J Immunol. 2013;43(3):805-14.
220. Sathkumara HD, De Silva NR, Handunnetti S, De Silva AD. Genetics of common variable immunodeficiency: role of transmembrane activator and calcium modulator and cyclophilin ligand interactor. Int J Immunogenet. 2015;42(4):239-53.
221. Romberg N, Chamberlain N, Saadoun D, et al. CVID-associated TACI mutations affect autoreactive B cell selection and activation. J Clin Invest. 2013;123(10):4283-93.
222. Losi CG, Silini A, Fiorini C, et al. Mutational analysis of human BAFF receptor TNFRSF13C (BAFF-R) in patients with common variable immunodeficiency. J Clin Immunol. 2005;25(5):496-502.
223. Kienzler AK, Salzer U, Warnatz K, et al. Human BAFF-R Deficiency is Associated with Primary Antibody Deficiency Syndrome. Clin Immunol. 2009;131:S123.
224. Germinaro M, Reynolds P, Knight V, Alam R. Association of B-cell activating factor receptor deficiency with the P21R polymorphism and common variable immunodeficiency. Ann Allergy Asthma Immunol. 2015;115(1):82-3.
225. Lougaris V, Baronio M, Moratto D, Cardinale F, Plebani A. Monoallelic BAFFR P21R/H159Y Mutations and Familiar Primary Antibody Deficiencies. J Clin Immunol. 2016;36(1):1-3.
226. Hildebrand JM, Luo Z, Manske MK, et al. A BAFF-R mutation associated with non-Hodgkin lymphoma alters TRAF recruitment and reveals new insights into BAFF-R signaling. J Exp Med. 2010;207(12):2569-79.
227. Wang HY, Ma CA, Zhao Y, et al. Antibody deficiency associated with an inherited autosomal dominant mutation in TWEAK. Proc Natl Acad Sci U S A. 2013;110(13):5127-32.
228. van Zelm MC, Reisli I, van der Burg M, et al. An antibody-deficiency syndrome due to mutations in the CD19 gene. N Engl J Med. 2006;354(18):1901-12.
229. van Zelm MC, Smet J, Adams B, et al. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. J Clin Invest. 2010;120(4):1265-74.
230. Thiel J, Kimmig L, Salzer U, et al. Genetic CD21 deficiency is associated with hypogammaglobulinemia. J Allergy Clin Immunol. 2012;129(3):801-810.e6.
231. Kanegane H, Agematsu K, Futatani T, et al. Novel mutations in a Japanese patient with CD19 deficiency. Genes Immun. 2007;8(8):663-70.
232. Conley ME, Dobbs AK, Farmer DM, et al. Primary B cell immunodeficiencies: comparisons and contrasts. Annu Rev Immunol. 2009;27:199-227.
233. Artac H, Reisli I, Kara R, et al. B-cell maturation and antibody responses in individuals carrying a mutated CD19 allele. Genes Immun. 2010;11(7):523-30.
234. van Zelm MC, Smet J, van der Burg M, et al. Antibody deficiency due to a missense mutation in CD19 demonstrates the importance of the conserved tryptophan 41 in immunoglobulin superfamily domain formation. Hum Mol Genet. 2011;20(9):1854-63.
235. Vince N, Boutboul D, Mouillot G, et al. Defects in the CD19 complex predispose to glomerulonephritis, as well as IgG1 subclass deficiency. J Allergy Clin Immunol. 2011;127(2):538-541.e1-5.
236. Skendros P, Rondeau S, Chateil JF, et al. Misdiagnosed CD19 deficiency leads to severe lung disease. Pediatr Allergy Immunol. 2014;25(6):603-6.
237. Vences-Catalan F, Kuo CC, Sagi Y, et al. A mutation in the human tetraspanin CD81 gene is expressed as a truncated protein but does not enable CD19 maturation and cell surface expression. J Clin Immunol. 2015;35(3):254-63.
238. van Zelm MC, Bartol SJ, Driessen GJ, et al. Human CD19 and CD40L deficiencies impair antibody selection and differentially affect somatic hypermutation. J Allergy Clin Immunol. 2014;134(1):135-44.
239. Wentink MW, Lambeck AJ, van Zelm MC, et al. CD21 and CD19 deficiency: Two defects in the same complex leading to different disease modalities. Clin Immunol. 2015;161(2):120-7.

240. Kuijpers TW, Bende RJ, Baars PA, et al. CD20 deficiency in humans results in impaired T cell-independent antibody responses. J Clin Invest. 2010;120(1):214-22.
241. van Montfrans JM, Hoepelman AI, Otto S, et al. CD27 deficiency is associated with combined immunodeficiency and persistent symptomatic EBV viremia. J Allergy Clin Immunol. 2012;129(3):787-793.e6.
242. Alkhairy OK, Perez-Becker R, Driessen GJ, et al. Novel mutations in TNFRSF7/CD27: Clinical, immunologic, and genetic characterization of human CD27 deficiency. J Allergy Clin Immunol. 2015;136(3):703-712.e10.
243. Salzer E, Daschkey S, Choo S, et al. Combined immunodeficiency with life-threatening EBV-associated lymphoproliferative disorder in patients lacking functional CD27. Haematologica. 2013;98(3):473-8.
244. Kotlarz D, Zietara N, Milner JD, Klein C. Human IL-21 and IL-21R deficiencies: two novel entities of primary immunodeficiency. Curr Opin Pediatr. 2014;26(6):704-12.
245. Salzer E, Kansu A, Sic H, et al. Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL-21 deficiency. J Allergy Clin Immunol. 2014;133(6):1651-9.e12.
246. Kotlarz D, Zietara N, Uzel G, et al. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. J Exp Med. 2013;210(3):433-43.
247. Stepensky P, Keller B, Abuzaitoun O, et al. Extending the clinical and immunological phenotype of human interleukin-21 receptor deficiency. Haematologica. 2015;100(2):e72-6.
248. Erman B, Bilic I, Hirschmugl T, et al. Combined immunodeficiency with CD4 lymphopenia and sclerosing cholangitis caused by a novel loss-of-function mutation affecting IL21R. Haematologica. 2015;100(6):e216-9.
249. Lopez-Herrera G, Tampella G, Pan-Hammarstrom Q, et al. Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. Am J Hum Genet. 2012;90(6):986-1001.
250. Kuehn HS, Ouyang W, Lo B, et al. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. Science. 2014;345(6204):1623-7.
251. Schubert D, Bode C, Kenefeck R, et al. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. Nat Med. 2014;20(12):1410-6.
252. Lo B, Zhang K, Lu W, et al. AUTOIMMUNE DISEASE. Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. Science. 2015;349(6246):436-40.
253. Alangari A, Alsultan A, Adly N, et al. LPS-responsive beige-like anchor (LRBA) gene mutation in a family with inflammatory bowel disease and combined immunodeficiency. J Allergy Clin Immunol. 2012;130(2):481-8.e2.
254. Burns SO, Zenner HL, Plagnol V, et al. LRBA gene deletion in a patient presenting with autoimmunity without hypogammaglobulinemia. J Allergy Clin Immunol. 2012;130(6):1428-32.
255. Charbonnier LM, Janssen E, Chou J, et al. Regulatory T-cell deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like disorder caused by loss-of-function mutations in LRBA. J Allergy Clin Immunol. 2015;135(1):217-27.
256. Revel-Vilk S, Fischer U, Keller B, et al. Autoimmune lymphoproliferative syndrome-like disease in patients with LRBA mutation. Clin Immunol. 2015;159(1):84-92.
257. Seidel MG, Hirschmugl T, Gamez-Diaz L, et al. Long-term remission after allogeneic hematopoietic stem cell transplantation in LPS-responsive beige-like anchor (LRBA) deficiency. J Allergy Clin Immunol. 2015;135(5):1384-90.e1-8.
258. Serwas NK, Kansu A, Santos-Valente E, et al. Atypical manifestation of LRBA deficiency with predominant IBD-like phenotype. Inflamm Bowel Dis. 2015;21(1):40-7.
259. Gamez-Diaz L, August D, Stepensky P, et al. The extended phenotype of LPS-responsive beige-like anchor protein (LRBA) deficiency. J Allergy Clin Immunol. 2016;137(1):223-30.
260. Sari S, Dogu F, Hwa V, et al. A Successful HSCT in a Girl with Novel LRBA Mutation with Refractory Celiac Disease. J Clin Immunol. 2016;36(1):8-11.
261. Alkhairy OK, Abolhassani H, Rezaei N, et al. Spectrum of Phenotypes Associated with Mutations in LRBA. J Clin Immunol. 2016;36(1):33-45.

262. Schreiner F, Plamper M, Dueker G, et al. Infancy-onset T1DM, short stature and severe immunodysregulation in two siblings with a homozygous LRBA-mutation. J Clin Endocrinol Metab. 2016;101(3):898-904.
263. Al-Mayouf SM, Naji H, Alismail K, et al. Evolving spectrum of LRBA deficiency-associated chronic arthritis: is there a causative role in juvenile idiopathic arthritis? Clin Exp Rheumatol. 2017;35(2):327-29.
264. Bakhtiar S, Gamez-Diaz L, Jarisch A, et al. Treatment of Infantile Inflammatory Bowel Disease and Autoimmunity by Allogeneic Stem Cell Transplantation in LPS-Responsive Beige-Like Anchor Deficiency. Front Immunol. 2017;8:52.
265. Bakhtiar S, Ruemmele F, Charbit-Henrion F, et al. Atypical Manifestation of LPS-Responsive Beige-Like Anchor Deficiency Syndrome as an Autoimmune Endocrine Disorder without Enteropathy and Immunodeficiency. Front Pediatr. 2016;4:98.
266. Shokri S, Nabavi M, Hirschmugl T, et al. LPS-Responsive Beige-Like Anchor Gene Mutation Associated With Possible Bronchiolitis Obliterans Organizing Pneumonia Associated With Hypogammaglobulinemia and Normal IgM Phenotype and Low Number of B Cells. Acta Med Iran. 2016;54(10):620-3.
267. Tesi B, Priftakis P, Lindgren F, et al. Successful Hematopoietic Stem Cell Transplantation in a Patient with LPS-Responsive Beige-Like Anchor (LRBA) Gene Mutation. J Clin Immunol. 2016;36(5):480-9.
268. Hayakawa S, Okada S, Tsumura M, et al. A Patient with CTLA-4 Haploinsufficiency Presenting Gastric Cancer. J Clin Immunol. 2016;36(1):28-32.
269. Zeissig S, Petersen BS, Tomczak M, et al. Early-onset Crohn's disease and autoimmunity associated with a variant in CTLA-4. Gut. 2015;64(12):1889-97.
270. Slatter MA, Engelhardt KR, Burroughs LM, et al. Hematopoietic stem cell transplantation for CTLA4 deficiency. J Allergy Clin Immunol. 2016;138(2):615-619.e1.
271. Lee S, Moon JS, Lee CR, et al. Abatacept alleviates severe autoimmune symptoms in a patient carrying a de novo variant in CTLA-4. J Allergy Clin Immunol. 2016;137(1):327-30.
272. Salzer E, Santos-Valente E, Klaver S, et al. B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C delta. Blood. 2013;121(16):3112-6.
273. Angulo I, Vadas O, Garcon F, et al. Phosphoinositide 3-kinase delta gene mutation predisposes to respiratory infection and airway damage. Science. 2013;342(6160):866-71.
274. Lucas CL, Kuehn HS, Zhao F, et al. Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency. Nat Immunol. 2014;15(1):88-97.
275. Kuehn HS, Boisson B, Cunningham-Rundles C, et al. Loss of B Cells in Patients with Heterozygous Mutations in IKAROS. N Engl J Med. 2016;374(11):1032-43.
276. Keller MD, Pandey R, Li D, et al. Mutation in IRF2BP2 is responsible for a familial form of common variable immunodeficiency disorder. J Allergy Clin Immunol. 2016;138(2):544-550.e4.
277. Belot A, Kasher PR, Trotter EW, et al. Protein kinase cdelta deficiency causes mendelian systemic lupus erythematosus with B cell-defective apoptosis and hyperproliferation. Arthritis Rheum. 2013;65(8):2161-71.
278. Kuehn HS, Niemela JE, Rangel-Santos A, et al. Loss-of-function of the protein kinase C delta (PKCdelta) causes a B-cell lymphoproliferative syndrome in humans. Blood. 2013;121(16):3117-25.
279. Kiykim A, Ogulur I, Baris S, et al. Potentially Beneficial Effect of Hydroxychloroquine in a Patient with a Novel Mutation in Protein Kinase Cdelta Deficiency. J Clin Immunol. 2015;35(6):523-6.
280. Ombrello MJ, Remmers EF, Sun G, et al. Cold urticaria, immunodeficiency, and autoimmunity related to PLCG2 deletions. N Engl J Med. 2012;366(4):330-8.
281. Zhou Q, Lee GS, Brady J, et al. A hypermorphic missense mutation in PLCG2, encoding phospholipase Cgamma2, causes a dominantly inherited autoinflammatory disease with immunodeficiency. Am J Hum Genet. 2012;91(4):713-20.
282. Chen K, Coonrod EM, Kumanovics A, et al. Germline mutations in NFKB2 implicate the noncanonical NF-kappaB pathway in the pathogenesis of common variable immunodeficiency. Am J Hum Genet. 2013;93(5):812-24.

283. Fliegauf M, V LB, Frede N, et al. Haploinsufficiency of the NF-kappaB1 Subunit p50 in Common Variable Immunodeficiency. Am J Hum Genet. 2015;97(3):389-403.
284. Brue T, Quentien MH, Khetchoumian K, et al. Mutations in NFKB2 and potential genetic heterogeneity in patients with DAVID syndrome, having variable endocrine and immune deficiencies. BMC Med Genet. 2014;15:139.
285. Lee CE, Fulcher DA, Whittle B, et al. Autosomal-dominant B-cell deficiency with alopecia due to a mutation in NFKB2 that results in nonprocessable p100. Blood. 2014;124(19):2964-72.
286. Lindsley AW, Qian Y, Valencia CA, Shah K, Zhang K, Assa'ad A. Combined immune deficiency in a patient with a novel NFKB2 mutation. J Clin Immunol. 2014 Nov;34(8):910-5.
287. Liu Y, Hanson S, Gurugama P, Jones A, Clark B, Ibrahim MA. Novel NFKB2 Mutation in Early-Onset CVID. J Clin Immunol. 2014;34(6):686-90.
288. Lougaris V, Tabellini G, Vitali M, et al. Defective natural killer-cell cytotoxic activity in NFKB2-mutated CVID-like disease. J Allergy Clin Immunol. 2015;135(6):1641-3.
289. Shi C, Wang F, Tong A, et al. NFKB2 mutation in common variable immunodeficiency and isolated adrenocorticotropic hormone deficiency: A case report and review of literature. Medicine (Baltimore). 2016;95(40):e5081.
290. Boztug H, Hirschmugl T, Holter W, et al. NF-kappaB1 Haploinsufficiency Causing Immunodeficiency and EBV-Driven Lymphoproliferation. J Clin Immunol. 2016;36(6):533-40.
291. Kaustio M, Haapaniemi E, Goos H, et al. Damaging heterozygous mutations in NFKB1 lead to diverse immunologic phenotypes. J Allergy Clin Immunol. 2017. pii: S0091-6749(17)30053-2.
292. Lougaris V, Moratto D, Baronio M, et al. Early and late B-cell developmental impairment in nuclear factor kappa B, subunit 1-mutated common variable immunodeficiency disease. J Allergy Clin Immunol. 2017;139(1):349-352.e1.
293. Schipp C, Nabhani S, Bienemann K, et al. Specific antibody deficiency and autoinflammatory disease extend the clinical and immunological spectrum of heterozygous NFKB1 loss-of-function mutations in humans. Haematologica. 2016;101(10):e392-e396.
294. Karban AS, Okazaki T, Panhuysen CI, et al. Functional annotation of a novel NFKB1 promoter polymorphism that increases risk for ulcerative colitis. Hum Mol Genet. 2004;13(1):35-45.
295. Yenmis G, Oner T, Cam C, et al. Association of NFKB1 and NFKBIA polymorphisms in relation to susceptibility of Behcet's disease. Scand J Immunol. 2015;81(1):81-6.
296. Jou ST, Chien YH, Yang YH, et al. Identification of variations in the human phosphoinositide 3-kinase p110delta gene in children with primary B-cell immunodeficiency of unknown aetiology. Int J Immunogenet. 2006;33(5):361-9.
297. Crank MC, Grossman JK, Moir S, et al. Mutations in PIK3CD Can Cause Hyper IgM Syndrome (HIGM) Associated with Increased Cancer Susceptibility. J Clin Immunol. 2014;34(3):272-6.
298. Hartman HN, Niemela J, Hintermeyer MK, et al. Gain of Function Mutations of PIK3CD as a Cause of Primary Sclerosing Cholangitis. J Clin Immunol. 2015;35(1):11-4.
299. Kracker S, Curtis J, Ibrahim MAA, et al. Occurrence of B-cell lymphomas in patients with activated phosphoinositide 3-kinase delta syndrome. J Allergy Clin Immunol. 2014;134(1):233-6.
300. Chiriaco M, Brigida I, Ariganello P, et al. A case of APDS patient: defects in maturation and function and decreased in vitro anti-mycobacterial activity in the myeloid compartment. Clin Immunol. 2015. pii: S1521-6616(15)30077-2.
301. Elgizouli M, Lowe DM, Speckmann C, et al. Activating PI3Kdelta mutations in a cohort of 669 patients with primary immunodeficiency. Clin Exp Immunol. 2016;183(2):221-9.
302. Coulter TI, Chandra A, Bacon CM, et al. Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: A large patient cohort study. J Allergy Clin Immunol. 2017;139(2):597-606.e4.
303. Teranishi H, Ishimura M, Koga Y, et al. Activated phosphoinositide 3-kinase delta syndrome presenting with gut-associated T-cell lymphoproliferative disease. Rinsho Ketsueki. 2017;58(1):20-25.
304. Tsujita Y, Mitsui-Sekinaka K, Imai K, et al. Phosphatase and tensin homolog (PTEN) mutation can cause activated phosphatidylinositol 3-kinase delta syndrome-like immunodeficiency. J Allergy Clin Immunol. 2016;138(6):1672-1680.e10.
305. Deau MC, Heurtier L, Frange P, et al. A human immunodeficiency caused by mutations in the PIK3R1 gene. J Clin Invest. 2014;124(9):3923-8.

306. Lucas CL, Zhang Y, Venida A, et al. Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K. J Exp Med. 2014;211(13):2537-47.
307. Kuhlen M, Honscheid A, Loizou L, et al. De novo PIK3R1 gain-of-function with recurrent sinopulmonary infections, long-lasting chronic CMV-lymphadenitis and microcephaly. Clin Immunol. 2016;162:27-30.
308. Lougaris V, Faletra F, Lanzi G, et al. Altered germinal center reaction and abnormal B cell peripheral maturation in PI3KR1-mutated patients presenting with HIGM-like phenotype. Clin Immunol. 2015;159(1):33-6.
309. Olbrich P, Lorenz M, Cura Daball P, et al. Activated PI3Kdelta syndrome type 2: Two patients, a novel mutation, and review of the literature. Pediatr Allergy Immunol. 2016;27(6):640-4.
310. Petrovski S, Parrott RE, Roberts JL, et al. Dominant Splice Site Mutations in PIK3R1 Cause Hyper IgM Syndrome, Lymphadenopathy and Short Stature. J Clin Immunol. 2016;36(5):462-71.
311. Elkaim E, Neven B, Bruneau J, et al. Clinical and immunologic phenotype associated with activated phosphoinositide 3-kinase delta syndrome 2: A cohort study. J Allergy Clin Immunol. 2016;138(1):210-218.e9.
312. Conley ME, Dobbs AK, Quintana AM, et al. Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K. J Exp Med. 2012;209(3):463-70.
313. Avila M, Dyment DA, Sagen JV, et al. Clinical reappraisal of SHORT syndrome with PIK3R1 mutations: towards recommendation for molecular testing and management. Clin Genet. 2015 Oct 24. [Epub ahead of print].
314. Capitani N, Ariani F, Amedei A, et al. Vav1 haploinsufficiency in a common variable immunodeficiency patient with defective T-cell function. Int J Immunopathol Pharmacol. 2012;25(3):811-7.
315. Alkhairy OK, Rezaei N, Graham RR, et al. RAC2 loss-of-function mutation in 2 siblings with characteristics of common variable immunodeficiency. J Allergy Clin Immunol. 2015;135(5):1380-4.e1-5.
316. Compeer EB, Janssen W, van Royen-Kerkhof A, van Gijn M, van Montfrans JM, Boes M. Dysfunctional BLK in common variable immunodeficiency perturbs B-cell proliferation and ability to elicit antigen-specific CD4+ T-cell help. Oncotarget. 2015;6(13):10759-71.
317. Goldman FD, Gurel Z, Al-Zubeidi D, et al. Congenital pancytopenia and absence of B lymphocytes in a neonate with a mutation in the Ikaros gene. Pediatr Blood Cancer. 2012;58(4):591-7.
318. Hoshino A, Okada S, Yoshida K, et al. Abnormal hematopoiesis and autoimmunity in human subjects with germline IKZF1 mutations. J Allergy Clin Immunol. 2016. pii: S0091-6749(16)31273-8.
319. Chou J, Lutskiy M, Tsitsikov E, Notarangelo LD, Geha RS, Dioun A. Presence of hypogammaglobulinemia and abnormal antibody responses in GATA2 deficiency. J Allergy Clin Immunol. 2014;134(1):223-6.
320. Kobbe R, Kolster M, Fuchs S, et al. Common variable immunodeficiency, impaired neurological development and reduced numbers of T regulatory cells in a 10-year-old boy with a STAT1 gain-of-function mutation. Gene. 2016;586(2):234-8.
321. Driessen GJ, H IJ, Wentink M, et al. Increased PI3K/Akt activity and deregulated humoral immune response in human PTEN deficiency. J Allergy Clin Immunol. 2016;138(6):1744-1747.e5.
322. Buchbinder D, Baker R, Lee YN, et al. Identification of patients with RAG mutations previously diagnosed with common variable immunodeficiency disorders. J Clin Immunol. 2015;35(2):119-24.
323. Volk T, Pannicke U, Reisli I, et al. DCLRE1C (ARTEMIS) mutations causing phenotypes ranging from atypical severe combined immunodeficiency to mere antibody deficiency. Hum Mol Genet. 2015;24(25):7361-72.
324. Abolhassani H, Wang N, Aghamohammadi A, et al. A hypomorphic recombination-activating gene 1 (RAG1) mutation resulting in a phenotype resembling common variable immunodeficiency. J Allergy Clin Immunol. 2014;134(6):1375-80.

325. Abolhassani H, Cheraghi T, Rezaei N, Aghamohammadi A, Hammarstrom L. Common Variable Immunodeficiency or Late-Onset Combined Immunodeficiency: A New Hypomorphic JAK3 Patient and Review of the Literature. J Investig Allergol Clin Immunol. 2015;25(3):218-20.
326. Chandra A, Zhang F, Gilmour KC, et al. Common variable immunodeficiency and natural killer cell lymphopenia caused by Ets-binding site mutation in the IL-2 receptor gamma (IL2RG) gene promoter. J Allergy Clin Immunol. 2016;137(3):940-2.e4.
327. Toubiana J, Okada S, Hiller J, et al. Heterozygous STAT1 gain-of-function mutations underlie an unexpectedly broad clinical phenotype. Blood. 2016;127(25):3154-64.
328. Browning MJ, Chandra A, Carbonaro V, Okkenhaug K, Barwell J. Cowden's syndrome with immunodeficiency. J Med Genet. 2015;52(12):856-9.
329. Heindl M, Handel N, Ngeow J, et al. Autoimmunity, intestinal lymphoid hyperplasia, and defects in mucosal B-cell homeostasis in patients with PTEN hamartoma tumor syndrome. Gastroenterology. 2012;142(5):1093-1096.e6.
330. Di Pierro V, Zuntini R, Cancrini C, et al. Consanguinity and polygenic diseases: a model for antibody deficiencies. Hum Hered. 2014;77(1-4):144-9.
331. Orange JS, Glessner JT, Resnick E, et al. Genome-wide association identifies diverse causes of common variable immunodeficiency. J Allergy Clin Immunol. 2011;127(6):1360-7.e6.
332. Li J, Wei Z, Li YR, et al. Understanding the genetic and epigenetic basis of common variable immunodeficiency disorder through omics approaches. Biochim Biophys Acta. 2016;1860(11 Pt B):2656-63.
333. Rodriguez-Cortez VC, Del Pino-Molina L, Rodriguez-Ubreva J, et al. Monozygotic twins discordant for common variable immunodeficiency reveal impaired DNA demethylation during naive-to-memory B-cell transition. Nat Commun. 2015;6:7335.
334. Offer SM, Pan-Hammarstrom Q, Hammarstrom L, Harris RS. Unique DNA repair gene variations and potential associations with the primary antibody deficiency syndromes IgAD and CVID. PLoS One. 2010;5(8):e12260.
335. Flinsenberg TW, Janssen WJ, Herczenik E, et al. A novel FcgammaRIIa Q27W gene variant is associated with common variable immune deficiency through defective FcgammaRIIa downstream signaling. Clin Immunol. 2014;155(1):108-17.
336. Packwood K, Drewe E, Staples E, et al. NOD2 polymorphisms in clinical phenotypes of common variable immunodeficiency disorders. Clin Exp Immunol. 2010;161(3):536-41.
337. Bellacchio E, Palma A, Corrente S, et al. The possible implication of the S250C variant of the autoimmune regulator protein in a patient with autoimmunity and immunodeficiency: in silico analysis suggests a molecular pathogenic mechanism for the variant. Gene. 2014;549(2):286-94.
338. Borte S, Celiksoy MH, Menzel V, et al. Novel NLRP12 mutations associated with intestinal amyloidosis in a patient diagnosed with common variable immunodeficiency. Clin Immunol. 2014;154(2):105-11.
339. Vorechovsky I, Cullen M, Carrington M, Hammarstrom L, Webster AD. Fine mapping of IGAD1 in IgA deficiency and common variable immunodeficiency: identification and characterization of haplotypes shared by affected members of 101 multiple-case families. J Immunol. 2000;164(8):4408-16.
340. Liao M, Ye F, Zhang B, et al. Genome-wide association study identifies common variants at TNFRSF13B associated with IgG level in a healthy Chinese male population. Genes Immun. 2012;13(6):509-13.
341. Cunningham-Rundles C. How I treat common variable immune deficiency. Blood. 2010;116(1):7-15.
342. Rizzi M, Neumann C, Fielding AK, et al. Outcome of allogeneic stem cell transplantation in adults with common variable immunodeficiency. J Allergy Clin Immunol. 2011;128(6):1371-1374.e2.

Chapter 2

Objectives

2 OBJECTIVES

The **general aim of this thesis** was to utilize detailed immunological phenotyping to facilitate the identification of molecular genetic defects in pediatric and adult patients with a diagnosis of common variable immunodeficiency (CVID) or overlapping primary antibody deficiencies (PADs) followed in our center. As indicated in the introduction, a conclusive genetic diagnosis holds great importance in terms of patient management and the elucidation of underlying disease mechanisms. Moreover, since the genetic defects described thus far only encompass 2-10% of the CVID population, novel mutations in known CVID genes and novel candidate disease genes were assumed to be found.

Objective 1. Clinical and immunological characterization of the study cohort.

Although the clinical and immunological phenotype of CVID had already been extensively studied, reports on related PADs such as idiopathic primary hypogammaglobulinemia (IPH) and IgG subclass deficiency (IgGSD) were limited. In **chapter 3**, we examined the clinical features of the CVID, IPH and IgGSD patients from our cohort and performed detailed immunophenotyping of peripheral blood mononuclear cells (PBMCs) in these patients in comparison with their asymptomatic first-degree family members and unrelated age-matched healthy controls. Based on these data, we aimed to (1) phenotypically characterize our study cohort, (2) improve current understanding on immunological abnormalities in milder PAD disorders like IPH and IgGSD, and (3) discriminate subgroups in the patient population by using a more comprehensive range of immunophenotypical parameters.

Objective 2. Genetic characterization of the study cohort.

Next, we aimed to identify the disease-causing genetic defects in our PAD patient cohort (70 index patients) by using one or more of the following methods: microarray-based comparative genomic hybridization (array CGH), targeted screening of candidate genes, whole exome sequencing (WES), and homozygosity mapping (in case of consanguinity). In **chapter 4**, it was our aim to compare the results of different genetic approaches and discuss the value of clinical and immunological characteristics to guide genetic assessment. We also proposed a strategy for genetic testing in patients with CVID and related antibody disorders, taking into account recent insights on a possible complex etiology in at least a subgroup of patients.

Additional objectives were formulated based on interesting genetic findings in specific patients.

Objective 3. Characterization of novel and rare mutations in known disease genes expanding phenotypical spectra.

In our patient cohort, we aimed to characterize novel and rare variants found in genes associated with either syndromic primary immunodeficiencies (*KMT2A*, *RNU4ATAC*) or CVID (*IKZF1*), expanding both the clinical and immunological spectrum of these three disorders. **Chapter 5** focused on familial cases of *KMT2A*-associated Wiedemann-Steiner syndrome and *RNU4ATAC*-associated Roifman syndrome, which exceptionally presented with a CVID-reminiscent phenotype in early childhood while extra-immunological features were not initially apparent. In **chapter 6**, we aimed to study a novel autosomal dominant *IKZF1* kindred with incomplete penetrance, and increase insights in early B cell development in symptomatic and asymptomatic mutation carriers.

Objective 4. Functional validation of novel candidate disease genes.

Finally, it was our objective to study novel candidate disease genes that we identified in our PAD cohort. In **chapter 7**, we described the discovery of novel compound heterozygous variants in *GTF3A* in two siblings with a clinical diagnosis of (possible) CVID. We aimed to present preliminary data from the initial functional validation studies and discuss plans for future research.

Chapter 3

The immunophenotypical fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives

3 THE IMMUNOPHENOTYPICAL FINGERPRINT OF PATIENTS WITH PRIMARY ANTIBODY DEFICIENCIES IS PARTIALLY PRESENT IN THEIR ASYMPTOMATIC FIRST-DEGREE RELATIVES.

Delfien J.A. Bogaert[1,2,3,4], Marieke De Bruyne[3], Veronique Debacker[1,5], Pauline Depuydt[3,6], Katleen De Preter[3,6], Carolien Bonroy[7], Jan Philippé[7,8], Victoria Bordon[9], Bart N. Lambrecht[4,10,11], Tessa Kerre[8,10,12], Andrea Cerutti[13,14], Karim Y. Vermaelen[5,10,11], Filomeen Haerynck[1,2,*] and Melissa Dullaers[1,4,10,*]

[1]Clinical Immunology Research Lab, Department of Pulmonary Medicine, Ghent University Hospital, Ghent, Belgium; [2]Department of Pediatric Immunology and Pulmonology, Centre for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, Belgium; [3]Center for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium; [4]Laboratory of Immunoregulation, VIB Inflammation Research Center, Ghent, Belgium; [5]Tumor Immunology Laboratory, Department of Pulmonary Medicine, Ghent University Hospital, Ghent, Belgium; [6]Cancer Research Institute Ghent, Ghent University, Belgium; [7]Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium; [8]Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Ghent, Belgium; [9]Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, Ghent University Hospital, Ghent, Belgium; [10]Department of Internal Medicine, Ghent University, Ghent, Belgium; [11]Department of Pulmonology, Ghent University Hospital, Ghent, Belgium; [12]Department of Hematology, Ghent University Hospital, Ghent, Belgium; [13]Department of Medicine, The Immunology Institute, Mount Sinai School of Medicine, New York, NY, USA; [14]B Cell Biology Laboratory, Hospital del Mar Medical Research Institute, Barcelona, Spain; [*]FH and MD contributed equally to this work.

Haematologica 2017;102(1):192-202. doi: 10.3324/haematol.2016.149112. Epub 2016 Sep 15.

ARTICLE

The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives

Delfien J.A. Bogaert,[1,2,3,4] Marieke De Bruyne,[1,3] Veronique Debacker,[1,5] Pauline Depuydt,[3,6] Katleen De Preter,[3,6] Carolien Bonroy,[7] Jan Philippé,[7,8] Victoria Bordon,[9] Bart N. Lambrecht,[4,10,11] Tessa Kerre,[8,10,12] Andrea Cerutti,[13,14] Karim Y. Vermaelen,[5,10,11] Filomeen Haerynck[1,2,*] and Melissa Dullaers[1,4,10,*]

[1]Clinical Immunology Research Laboratory, Department of Pulmonary Medicine, Ghent University Hospital, Belgium; [2]Department of Pediatric Immunology and Pulmonology, Centre for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Belgium; [3]Center for Medical Genetics, Ghent University and Ghent University Hospital, Belgium; [4]Laboratory of Immunoregulation, VIB Inflammation Research Center, Ghent, Belgium; [5]Tumor Immunology Laboratory, Department of Pulmonary Medicine, Ghent University Hospital, Belgium; [6]Cancer Research Institute, Ghent University, Belgium; [7]Department of Laboratory Medicine, Ghent University Hospital, Belgium; [8]Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Belgium; [9]Department of Pediatric Hematology, Oncology and Stem Cell Transplantation, Ghent University Hospital, Belgium; [10]Department of Internal Medicine, Ghent University, Belgium; [11]Department of Pulmonology, Ghent University Hospital, Belgium; [12]Department of Hematology, Ghent University Hospital, Belgium; [13]Department of Medicine, The Immunology Institute, Mount Sinai School of Medicine, New York, NY, USA and [14]B Cell Biology Laboratory, Hospital del Mar Medical Research Institute, Barcelona, Spain

FH and MD contributed equally to this work.

Haematologica 2017
Volume 102(1):192-202

Correspondence:
melissa.dullaers@ugent.be

Received: May 13, 2016.
Accepted: September 8, 2016.
Pre-published: September 15, 2016.

doi:10.3324/haematol.2016.149112

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/102/1/192

ABSTRACT

The etiology of primary antibody deficiencies is largely unknown. Beside rare monogenic forms, the majority of cases seem to have a more complex genetic basis. Whereas common variable immunodeficiency has been investigated in depth, there are only a few reports on milder primary antibody deficiencies such as idiopathic primary hypogammaglobulinemia and IgG subclass deficiency. We performed flow cytometric immunophenotyping in 33 patients with common variable immunodeficiency, 23 with idiopathic primary hypogammaglobulinemia and 21 with IgG subclass deficiency, as well as in 47 asymptomatic first-degree family members of patients and 101 unrelated healthy controls. All three groups of patients showed decreased memory B- and naïve T-cell subsets and decreased B-cell activating factor receptor expression. In contrast, circulating follicular helper T-cell frequency and expression of inducible T-cell costimulator and chemokine receptors were only significantly altered in patients with common variable immunodeficiency. Asymptomatic first-degree family members of patients demonstrated similar, albeit intermediate, alterations in naïve and memory B- and T-cell subsets. About 13% of asymptomatic relatives had an abnormal peripheral B-cell composition. Furthermore, asymptomatic relatives showed decreased levels of $CD4^+$ recent thymic emigrants and increased central memory T cells. Serum IgG and IgM levels were also significantly lower in asymptomatic relatives than in healthy controls. We conclude that, in our cohort, the immunophenotypic landscape of primary antibody deficiencies comprises a spectrum, in which some alterations are shared between all primary antibody deficiencies whereas others are only associated with common variable immunodeficiency. Importantly, asymptomatic first-degree family members of patients were found to have an intermediate phenotype for peripheral B- and T-cell subsets.

Introduction

Primary antibody deficiencies (PAD) are the most prevalent primary immune deficiencies and are characterized by impaired production of one or more immunoglobulin (Ig) isotypes. Since the description of Bruton agammaglobulinemia in 1952,[1] our understanding of PAD has improved substantially.[2] Nonetheless, the etiology of many PAD remains largely unknown.[2] Common variable immunodeficiency (CVID) is one of the most common PAD and is a clinically and immunologically heterogeneous disorder.[2,3] Indeed, the definition of CVID is a topic of ongoing debate. The term CVID was introduced in 1971 to distinguish less well-defined PAD from those with a consistent phenotype and inheritance.[4] In 1999, CVID was redefined by the European Society for Immunodeficiencies (ESID) and the Pan-American Group for Immunodeficiency (PAGID): a marked decrease in serum IgG with a marked decrease in serum IgM and/or IgA, poor antibody response to vaccines and/or absent isohemagglutinins, and exclusion of secondary or other defined causes of hypogammaglobulinemia.[5] About 15 years later, two different revisions of the ESID/PAGID 1999 criteria were made: the Ameratunga 2013 criteria[6] and the revised ESID registry 2014 criteria.[7] Remarkably, both revisions proposed reduced (switched) memory B cells as an alternative criterion for impaired vaccine responses.[7] The revised ESID registry 2014 criteria additionally stated that both IgG and IgA must be decreased to confer a diagnosis of CVID.[7] However, not all practitioners agree on the obligatory decrease in IgA.[3] In 2016, an international consensus statement on CVID proposed less stringent diagnostic criteria, closely resembling the ESID/PAGID 1999 criteria and not including a reduction in memory B cells.[3]

CVID patients have an increased susceptibility to infections, predominantly of the respiratory tract.[3,8] Moreover, they are prone to developing non-infectious complications such as autoimmunity, polyclonal lymphoproliferation, and malignancies.[3,8] Patients with hypogammaglobulinemia showing clinical features reminiscent of CVID but not fulfilling all laboratory criteria are often encountered in daily practice.[2,3] For the latter group of patients, consensus diagnostic criteria, prevalence rates and clinical and immunophenotypic data are scarce.[9] These patients are henceforth referred to as having idiopathic primary hypogammaglobulinemia (IPH),[9] although various other terminologies have also been used such as CVID-like disorders[10] and unclassified hypogammaglobulinemia.[11] Patients with a marked decrease in one or more IgG subclasses but normal total IgG are diagnosed with IgG subclass deficiency (IgGSD).[12] Since IgG1 constitutes 66% of total IgG, IgG1 deficiency typically results in decreased total IgG.[12] IgG4 only forms a minor portion of total IgG (3%), and isolated IgG4 deficiency is usually asymptomatic.[12] Patients with isolated IgG2 and/or IgG3 deficiency can suffer from recurrent infections and some develop non-infectious, especially autoimmune, complications.[12,13] However, subnormal Ig isotype levels and in particular subnormal IgG subclass levels are not always accompanied by a clinical phenotype.[2,13] On the other hand, milder PAD phenotypes can sometimes evolve into a complete CVID phenotype over time.[3]

There is increasing evidence that besides rare monogenic forms, the majority of PAD are complex disorders in which multiple genes and/or environmental factors determine the final phenotype.[3] This has been best documented for CVID.[14] A monogenic cause has only been identified in 2-10% of cases of CVID (e.g. *ICOS*, *CD19*, *CD21*), and in several families the same primary genotype resulted in a large phenotypic variability from asymptomatic over milder PAD forms to complete CVID (e.g. *TACI*, *BAFF-R*, *NFKB1*, *CTLA4*).[14] Furthermore, it has been recently reported that sporadic patients with CVID have variants in multiple CVID-associated genes and an enrichment of variants in pathways important in B-cell function, suggesting a polygenic nature of CVID.[15] In addition, B cells in CVID patients have been shown to have DNA methylation alterations in genes critical for B-cell function, implicating a role for epigenetic factors in the pathogenesis of CVID.[16]

While the immunological phenotype of CVID has been investigated in depth, there are only few reports on IPH and IgGSD and none on cohorts of asymptomatic PAD family members. We, therefore, performed a detailed immunophenotypic analysis of CVID, IPH and IgGSD patients as well as asymptomatic first-degree relatives of the PAD patients included in the study (asymptomatic family members, AFM) and unrelated healthy controls (HC). With this study, we demonstrate for the first time in a relatively large cohort that the spectrum of immunophenotypic alterations ranges from AFM through milder PAD entities, such as IPH and IgGSD, to the most severe entity, CVID, supporting the notion of a multifactorial origin of PAD.

Methods

Study population

The study populations consisted of 33 patients with CVID, 23 with IPH, 21 with IgGSD, 47 AFM, and 101 HC. Patients were recruited between October 2013 and November 2015 at the Departments of Pediatrics, Hematology and Pulmonology at Ghent University Hospital. Clinical data were retrospectively collected from patients' medical records. CVID was defined as decreased [from hereon always meaning: at least 2 standard deviations (SD) below the age-adjusted mean according to the local laboratory reference values, measured at least twice] IgG, decreased IgA and/or IgM, and poor antibody responses to protein and/or polysaccharide vaccines.[3] IPH was defined as decreased IgG, normal or decreased IgA and/or IgM, and good antibody responses to protein and/or polysaccharide vaccines. IgGSD was defined as decreased IgG2 and/or IgG3, normal total IgG and IgM, normal or decreased IgA, and good or poor antibody responses to protein and/or polysaccharide vaccines. Patients with other defined causes of antibody deficiency and/or profound T-cell defects, as determined by the ESID registry criteria for CVID (http://esid.org/Working-Parties/Registry/Diagnosis-criteria), were excluded from the study. At the time of analysis, 32 of 33 CVID patients, 21 of 23 of those with IPH, and 15 of 21 patients with IgGSD were receiving regular immunoglobulin replacement therapy. One of the CVID patients was being given tacrolimus and rapamycin as maintenance immunosuppressive drugs after liver transplantation; the remaining patients were not on immunosuppression.

AFM were defined as first-degree family members of included patients who did not suffer from recurrent infections, autoimmunity or other signs of immune deficiency or dysregulation. HC were recruited from hospital and university staff and their children. Exclusion criteria for HC were pregnancy, medical conditions that affect the immune system, and treatment with immuno-

suppressive or immune-modulating drugs. To obtain medical information regarding AFM and HC, a detailed clinical history was taken at the time of their inclusion in the study.

The study was approved by the ethical committee of Ghent University Hospital (2012/593). All reported subjects (or their parents in the case of pediatric subjects) provided written informed consent to participation in the study, in accordance with the Helsinki Declaration of 1975.

Serum immunoglobulins, flow cytometry, unsupervised computational clustering analysis, and statistics

Details on measurement of serum Ig levels and absolute white blood cell counts, flow cytometric analysis of peripheral blood mononuclear cells, unsupervised computational clustering methods, the statistical analysis and conversion into z-scores are provided in the *Online Supplementary Methods*.

Results

Characteristics of the study population

A comprehensive analysis was performed of the clinical and immunological phenotype of 77 patients with PAD (33 with CVID, 23 with IPH, and 21 with IgGSD), 47 AFM, and 101 HC. The demographics of the study population are shown in Table 1. One IPH patient was born from a consanguineous marriage; both parents were asymptomatic and included as AFM.

Median levels of IgG at diagnosis were significantly lower in CVID than in IPH (Figure 1A). As expected from the clinical definition, median IgG levels were also significantly lower in CVID than in IgGSD, and median IgA and IgM levels were significantly lower in CVID than in either IPH or IgGSD (Figure 1A). None of the HC or AFM had severely decreased Ig levels (i.e. z-score below 2.5), except for one AFM (mother of an IPH patient) who had a total IgG level of 3.5 SD below the age-adjusted mean (Figure 1A). Interestingly, mean IgG and IgM levels were significantly lower in AFM than in HC (Figure 1A).

Details on absolute white blood cell counts from routine laboratory evaluations are provided in the *Online Supplementary Results*, *Online Supplementary Figure S1* and *Online Supplementary Table S2*. Patients' clinical characteristics are summarized in *Online Supplementary Table S3*. At the time of analysis, all patients suffered from recurrent infections, predominantly of the respiratory tract. A higher proportion of IPH patients developed warts or fungal infections (8.7% and 17.4%, respectively) compared to CVID patients (3.0% and 3.0%, respectively) and IgGSD patients (0% and 9.5%, respectively), although these differences were not statistically significant (P=0.312 and P=0.184, respectively). Bronchiectasis was seen in 54.2% of CVID patients, 30.8% of IPH patients and 16.7% of IgGSD patients, which fits with their gradually milder defects in Ig production (*Online Supplementary Table S3*). The overall occurrence of non-infectious disease-related complications was significantly higher in CVID patients than in IPH patients (P=0.031), and also higher in patients with CVID than in those with IgGSD although in this case the difference did not reach statistical significance (P=0.070) (Figure 1B). In particular, autoimmune manifestations (symptoms related to autoimmune disease) and benign lymphadenopathy (cervical, mediastinal and/or abdominal lymph nodes >1 cm diameter, detected at least twice on medical imaging) were significantly greater in CVID than in IPH or IgGSD (Figure 1C).

Abnormalities in peripheral B-cell subsets

An abnormal distribution of peripheral B-cell subsets is one of the best-established features in CVID.[3] We, therefore, examined peripheral B-cell subsets extensively. A representative gating strategy is shown in *Online Supplementary Figure S2*. Mean total B-cell percentages were comparable between all groups of patients (*Online Supplementary Figure S3*), but absolute B-cell numbers were significantly lower in CVID than in IPH or IgGSD (*Online Supplementary Figure S1*). On average, the B-cell phenotype was most deviant in CVID with significantly decreased IgD^+CD27^+ marginal zone-like B cells, IgD^-CD27^+ memory B cells and plasmablasts and significantly increased naïve, transitional and $CD21^{low}$ B cells (Figure 2). Although differences did not always reach statistical significance, IPH and IgGSD patients as well as AFM showed a similar trend towards a decrease in antigen-experienced B-cell subsets and an increase in immature/naïve B-cell subsets (Figure 2). The levels of IgD^-CD27^+ memory B cells, in particular, were significantly decreased in all three groups of patients compared to the levels in HC. $IgD^-CD27^+IgG^+$ memory B cells were reduced in CVID and IPH but not in IgGSD, corresponding with the inclusion criteria. Remarkably, $IgD^-CD27^+IgG^+$ memory B-cell levels were also significantly lower in AFM than in HC (Figure 2). $IgD^-CD27^+IgA^+$ memory B-cell levels were significantly lower in CVID but unexpectedly higher in IgGSD than in HC (Figure 2). As expected, $IgD^-CD27^+IgG^+$ and IgA^+ memory B-cell levels showed strong positive correlations with serum IgG and IgA levels, respectively (both P<0.001, *data not shown*). Mean levels of $IgD^-CD27^+IgM^+$ memory B cells were significantly higher in CVID and IPH, probably reflecting the relative decrease in IgG^+ and IgA^+ memory B cells (*Online Supplementary Figure S3*). No significant differences were found in plasma cells or $IgM^+IgD^+CD27^+$ marginal zone-like B cells (*Online Supplementary Figure S3*).

Based on the composition of peripheral B-cell subsets, Driessen *et al.* distinguished five patterns indicating at what stage (early to late) in peripheral B-cell development a defect may be located, as explained in the legend to Figure 2B.[17] Here, study subjects were categorized using age-adjusted B-cell subset proportions (z-scores) instead of absolute counts. All HC and the majority of AFM, IPH and IgGSD showed a normal peripheral B-cell composition (pattern 5), whereas CVID patients were divided over the five patterns (Figure 2B). B-cell patterns 1 and 2 were only seen in CVID and IgGSD patients, whereas patterns 3 and 4 were observed in CVID, IPH, and IgGSD patients and, remarkably, even in AFM (Figure 2B). The distribution of B-cell patterns was significantly different in patient groups

Table 1. Demographics of the study population.

	Number (male/female)	Age at inclusion in years (mean, range)
HC	101 (44/57)	30.8 (4.5 - 82.2)
AFM	47 (23/24)	45.1 (6.2 - 71.0)
CVID	33 (19/14)	28.0 (7.7 - 83.2)
IPH	23 (9/14)	35.6 (10.3 - 86.5)
IgGSD	21 (3/18)	39.0 (11.9 - 65.0)

AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia.

and AFM compared to HC (all $P \leq 0.001$). In addition, B-cell patterns in CVID differed significantly from those in IPH, IgGSD and AFM (all $P<0.01$). B-cell patterns were not significantly different between IPH, IgGSD and AFM.

Abnormalities in B-cell co-stimulatory molecules and chemokine receptors

Defects in B-cell co-stimulatory molecules and chemokine receptors have been associated with CVID.[3] In this study we examined the expression of various co-stimulatory molecules [B-cell activating factor belonging to the TNF family - receptor (BAFF-R), human leukocyte antigen – antigen D-related (HLA-DR), cluster of differentiation 40 (CD40) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI)] and chemokine receptors [CXC-chemokine receptor 5 (CXCR5) and CC-chemokine receptor 7 (CCR7)].

Average BAFF-R expression on B cells was severely decreased in CVID and moderately decreased in IPH and IgGSD (Figure 2C). Mean HLA-DR expression on B cells

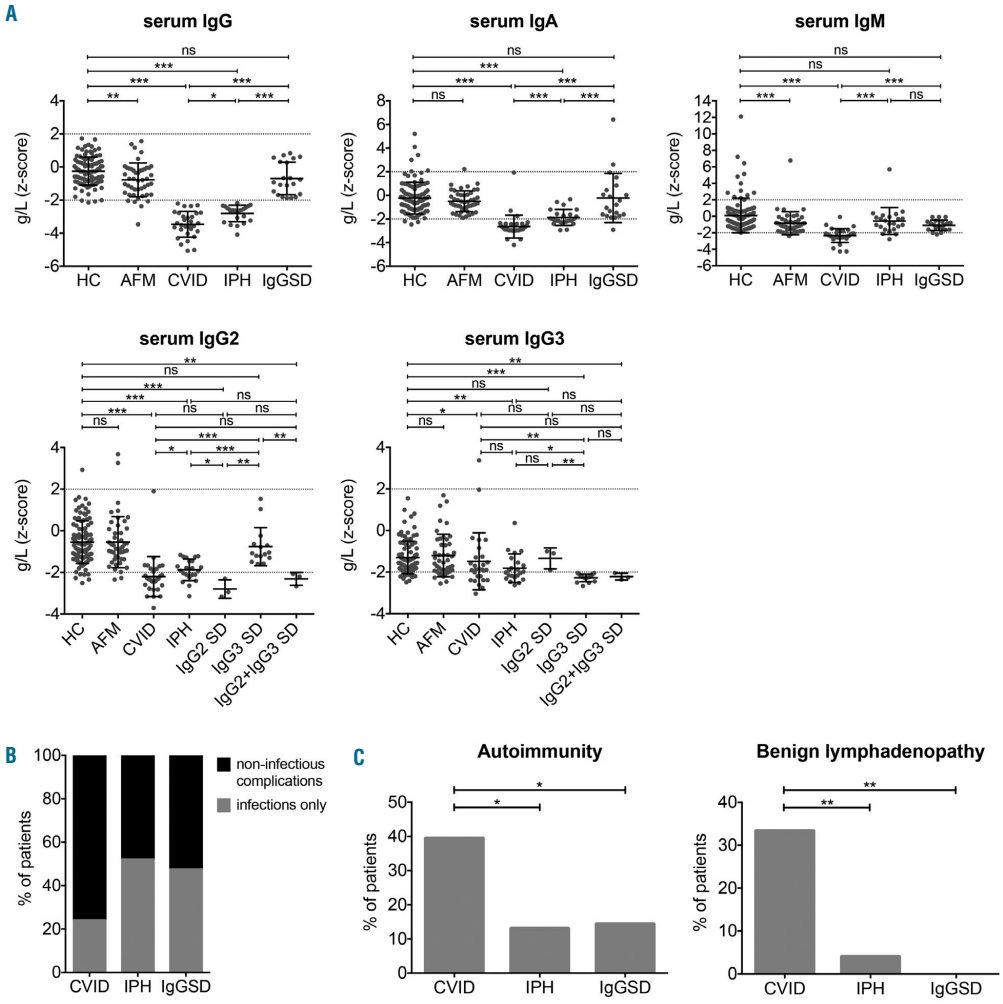


Figure 1. Serum immunoglobulin levels and clinical phenotype. (A) Serum immunoglobulin (Ig) levels at diagnosis (CVID, IPH, IgGSD) or at inclusion in the study (HC, AFM). For IgG2 and IgG3 levels, IgGSD patients were subdivided in accordance with the decreased IgG subclasses. Graphs represent mean ± SD. Ig values were expressed as z-scores to adjust for age. Values normal for age have a z-score between -2 and 2 (dotted lines). (B) The clinical phenotype of CVID, IPH and IgGSD patients. Non-infectious complications included benign lymphadenopathy, lymphocytic interstitial pneumonitis, granulomata, splenomegaly, hepatomegaly, unexplained enteropathy and/or autoimmunity. (C) Prevalence of autoimmune manifestations (symptoms related to autoimmune disease) and benign lymphadenopathy (cervical, mediastinal and/or abdominal lymph nodes >1 cm diameter, detected at least twice on medical imaging) in CVID, IPH, and IgGSD patients. AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons).

was markedly elevated in CVID and to a lesser extent in IgGSD (*Online Supplementary Figure S4*). All groups of patients had similarly increased mean CD40 expression on B cells compared to the mean expression on B cells from HC (*Online Supplementary Figure S4*). Mean TACI expression was lower in IgGSD but was not aberrant in the other groups of patients (*Online Supplementary Figure S4*). On average, AFM did not have deviant expression of the investigated co-stimulatory molecules (Figure 2C, *Online Supplementary Figure S4*).

CXCR5 and CCR7 are implicated in B- and T-cell migration to secondary lymphoid organs and in differential localization of these cells in follicles.[18] In our cohort, CXCR5 and CCR7 expression on B cells was severely reduced in CVID but not in the other groups of patients or in AFM (Figure 3A). Furthermore, CXCR5 and CCR7 expression on B cells was positively correlated with levels of IgD$^-$CD27$^+$ memory B cells, IgD$^-$CD27$^+$IgG$^+$ and IgA$^+$ memory B cells and IgD$^+$CD27$^+$ marginal zone-like B cells, and negatively correlated with CD21^{low} B-cell levels (Figure 3B).

Abnormalities in the T-cell compartment

Alongside B-cell abnormalities, multiple alterations have been described in the T-cell compartment of CVID patients.[3] *Online Supplementary Figure S5* depicts a representative gating strategy of the here-examined T-cell subsets.

CVID patients showed, on average, skewing towards memory T-cell subsets evidenced by significantly increased central memory T (TCM) cells and decreased naïve T cells and CD4$^+$ recent thymic emigrants (RTE) (Figure 4). A similar though less pronounced trend was also observed in AFM and patients with IPH: both groups

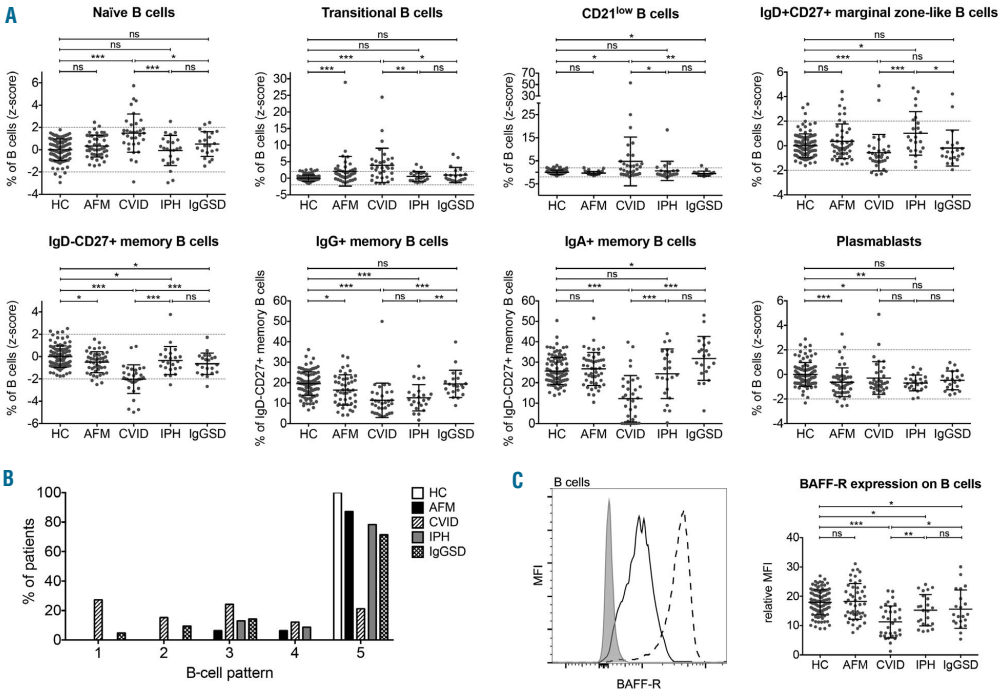


Figure 2. Peripheral B-cell subsets and BAFF-R expression on B cells. (A) Total B cells were gated as CD19$^+$CD20$^+$ in living cells. Within B cells, naïve B cells were gated as IgD$^+$CD27$^-$, transitional B cells as CD24^{high}CD38^{high}, CD21^{low} B cells as CD21^{low}CD38^{low}, marginal zone-like B cells as IgD$^+$CD27$^+$, memory B cells as IgD$^-$CD27$^+$, and plasmablasts as CD38^{high}CD24$^-$. Graphs represent mean ± SD. B-cell subsets were expressed as z-scores to adjust for age if applicable. Values normal for age have a z-score between -2 and 2 (dotted lines). (B) B-cell patterns according to Driessen *et al.*[17] using age-adjusted B-cell subset proportions (z-scores). B-cell pattern 1: reduced transitional and IgD$^-$CD27$^+$ memory B cells, indicative of a defect in B-cell production and germinal center function. B-cell pattern 2: normal transitional B cells and reduced naïve, IgD$^+$CD27$^+$ marginal zone-like and IgD$^-$CD27$^+$ memory B cells, indicative of a defect in early B-cell maturation or survival. B cell pattern 3: reduced IgD$^+$CD27$^+$ marginal zone-like and IgD$^-$CD27$^+$ memory B cells, indicative of a defect in B-cell activation and proliferation. B-cell pattern 4: isolated reduction of IgD$^-$CD27$^+$ memory B cells, indicative of a defect in germinal center function. B-cell pattern 5: normal IgD$^+$CD27$^+$ marginal zone-like and IgD$^-$CD27$^+$ memory B cells; in individuals with decreased serum immunoglobulin levels, this could be indicative of a post-germinal center defect. (C) BAFF-R expression on B cells. The graph represents mean ± SD. Representative flow cytometric analysis is shown on the left. The full black line represents a CVID patient, the dashed black line represents a HC. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population (black line) by the MFI of the "fluorescence minus one" (FMO) population (gray). AFM, asymptomatic family member; CVID, common variable immunodeficiency; HC, healthy control; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia. * P≤0.05, ** P≤0.01, *** P≤0.001, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons).

displayed significantly increased $CD8^+$ TCM cells, and AFM also had increased $CD4^+$ TCM cells and decreased $CD4^+$ RTE (Figure 4). In contrast, IgGSD patients showed skewing towards naïve T-cell subsets evidenced by significantly increased levels in naïve T cells and $CD4^+$ RTE, without significant differences in memory T-cell subsets (Figure 4).

$HLA–DR^+CD8^+$ T-cell counts were significantly elevated in CVID patients and AFM compared to HC. CVID patients also had higher numbers of $HLA–DR^+CD4^+$ T cells (*Online Supplementary Figure S6*). This is analogous to the increase in mean HLA-DR expression on B cells in CVID (*Online Supplementary Figure S4*). We found no important differences in regulatory T cells and double negative T cells (*Online Supplementary Figure S6*).

Follicular helper T (Tfh) cells orchestrate the formation of high-affinity antibody-producing plasma cells and memory B cells, and inducible T-cell co-stimulator (ICOS) is a key molecule in the differentiation and function of Tfh cells.[19] In our cohort, mean levels of circulating Tfh (cTfh) cells were significantly higher in CVID patients than in HC (Figure 5A). Within the CVID group, a higher proportion of cTfh cells expressed ICOS in the resting condition (Figure 5A). Upon stimulation, however, ICOS expression on $CD4^+$ T cells was markedly lower in CVID patients than in HC, despite adequate T-cell activation as evidenced by the normal CD69 upregulation (Figure 5B). A similarly decreased ICOS upregulation was also seen on $CD8^+$ T cells in CVID patients (*Online Supplementary Figure S7*). IPH and IgGSD patients and AFM did not demonstrate alterations in mean cTfh cell percentages or ICOS expression (Figure 5A,B).

cTfh cells in CVID patients displayed a highly significant reduction in mean CCR7 expression (Figure 5C). Additionally, CVID patients had decreased CCR7 expression on total T cells and naïve, RTE and central memory T-cell subsets (*Online Supplementary Figure S8*), analogous to the decrease in mean CCR7 expression levels on B cells (Figure 3). IPH and IgGSD patients and AFM demonstrated normal mean CCR7 expression on cTfh cells and naïve and memory T-cell subsets (Figure 5C and *Online Supplementary Figure S8*). Interestingly, CCR7 expression on cTfh cells was significantly correlated with the level of cTfh cells (Figure 5D). Furthermore, CCR7 expression on total T cells was positively correlated with levels of naïve T-cell subsets, and negatively correlated with levels of memory T-cell subsets except for $CD8^+$ TCM cells (*Online Supplementary Figure S9*).

Abnormalities in natural killer cells and dendritic cells

Besides B and T lymphocytes, innate immune cells have also been shown to be affected in CVID patients.[3] We, therefore, screened for relevant innate immune cell subsets (the gating strategy is shown in *Online Supplementary Figure S10*). On average, levels of total natural killer (NK) cells were significantly lower in all PAD groups compared

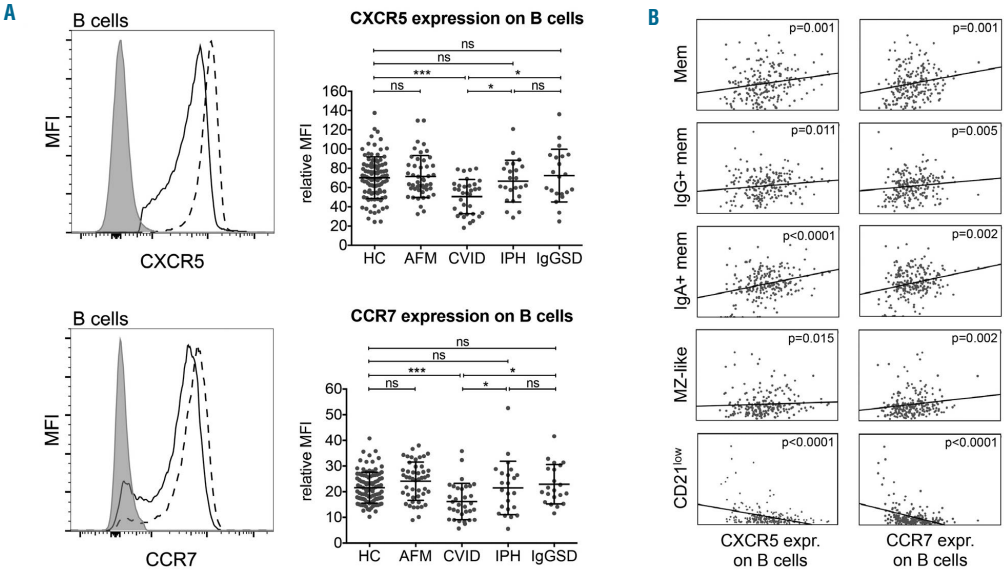


Figure 3. CXCR5 and CCR7 expression on B cells. (A) Graphs represent mean ± SD. Representative flow cytometric analysis is shown on the left. Full black lines represent CVID patients, dashed black lines represent HC. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population (black line) by the MFI of the "fluorescence minus one" (FMO) population (gray). AFM, asymptomatic family member; CVID, common variable immunodeficiency; HC, healthy control; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia. *P≤0.05, ***P≤0.001, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons). (B) Significant correlations between CXCR5 and CCR7 expression on B cells and levels of B-cell subsets. Correlations were calculated with the Spearman rank correlation. Expr, expression; mem, IgD^-CD27^+ memory B cells; MZ-like, IgD^-CD27^+ marginal zone-like B cells; $CD21^{low}$, $CD21^{low}$ B cells.

to the group of HC, while NK-cell subsets were only aberrant in CVID (Figure 6). In our cohort, CVID patients had relatively increased levels of CD56^{bright} NK cells (a more immature NK phenotype) and relatively decreased levels of CD16^{bright} NK cells (a more effector NK phenotype) (Figure 6). This suggests a more immature NK-cell profile in CVID patients, analogous to their immature B-cell profile but opposite to their exhausted T-cell profile. Mean NKT- and invariant NKT-cell percentages were similar across groups (Figure 6). The levels of dendritic cells were, however, significantly decreased in all groups of patients (Figure 6). The relative proportion of conventional and plasmacytoid dendritic cells was not significantly altered in any of the patients (Figure 6). On average, there were no significant differences in the examined innate immune cells between AFM and HC, contrasting with the findings in the B- and T-cell compartments (Figure 6).

Unsupervised computational clustering analysis

Since significant associations between clinical features and immunological parameters had been found (see *Online Supplementary Results* and *Online Supplementary Table S4*), we investigated whether subgroups of the study subjects could be distinguished based on immunophenotypic data. We performed unsupervised computational clustering analysis of flow cytometric parameters by means of hierarchical clustering with heatmaps and principal component analysis. We could neither distinguish entire patient groups from healthy groups, nor distinguish subgroups among patients stratified according to diagnosis or clinical phenotype (*Online Supplementary Figure S11*). Furthermore, there was no clustering according to age or between members of the same family (*data not shown*). Similarly, principal component analysis could not reveal patient subgroups from the immunological data and could not distinguish patients from AFM or HC (*data not shown*). Different combinations of immunological parameters or patient stratifications did not improve the clustering (*data not shown*).

Discussion

Here we show for the first time in a relatively large cohort of subjects that there is a spectrum of peripheral immunophenotypic abnormalities in PAD, ranging from those present in AFM across those found in IgGSD and IPH patients to those in CVID patients, with the differences being most notable in naïve and memory T- and B-lymphocyte subsets. A summary of significantly different parameters in the patient and AFM groups compared to

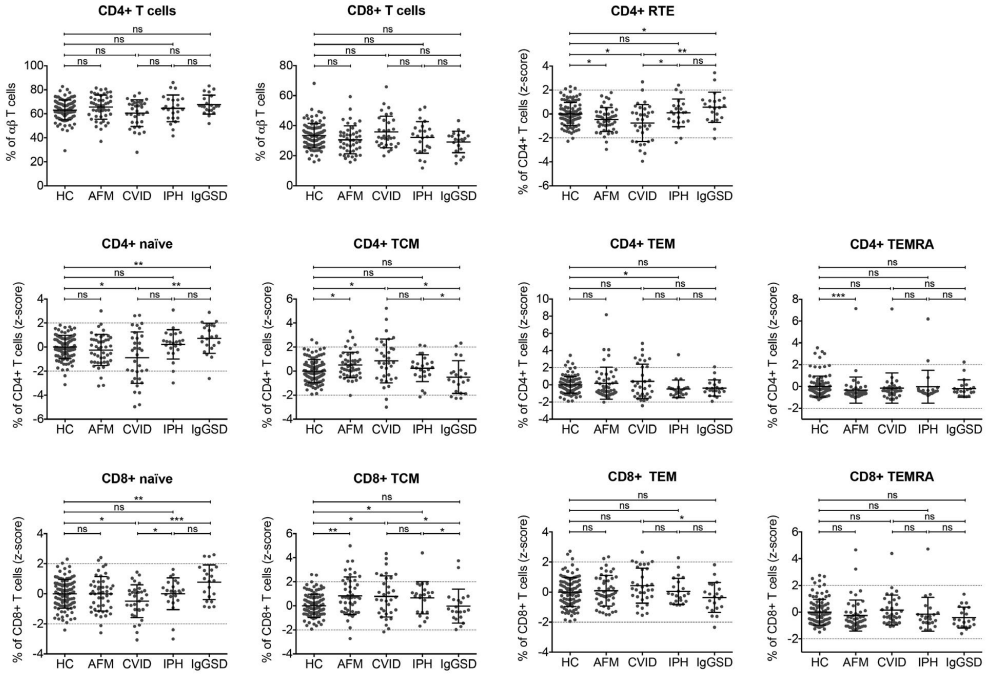


Figure 4. Naïve and memory T-cell subsets. Total T cells were gated as CD3$^+$ in alive cells. CD4$^+$ and CD8$^+$ T cells were gated in αβ T cells. Within CD4$^+$ and CD8$^+$ T cells, naïve cells were gated as CD45RO$^-$CCR7$^+$, central memory cells (TCM) as CD45RO$^+$CCR7$^+$, effector memory cells (TEM) as CD45RO$^+$CCR7$^-$, and terminally differentiated cells (TEMRA) as CD45RO$^-$CCR7$^-$. In naïve CD4$^+$ T cells, recent thymic emigrants (RTE) were gated as CD31$^+$. Graphs represent mean ± SD. Naïve and memory T-cell subsets were expressed as z-scores to adjust for age. Values normal for age have a z-score between -2 and 2 (dotted lines). AFM, asymptomatic family member; CVID, common variable immunodeficiency; HC, healthy control; IgGSD, IgG subclass deficiency; IPH, idiopathic primary hypogammaglobulinemia. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons).

the HC group is shown in Figure 7. The results presented support the notion of a complex basis of CVID and related milder PAD disorders, in which an accumulation of multiple genetic and/or environmental factors contributes to the final phenotype.[3,15,16] Monogenic defects have only been identified in a minority of cases with CVID.[14] Remarkably, some relatives with the same monogenic defect were found to be asymptomatic or suffer from a milder PAD phenotype such as IgGSD.[20-23] A multifactorial basis in the majority of CVID, and in extension PAD, would explain the vast variations in the clinical and immunological landscape seen in these patients.[2,14]

The immunophenotypic characteristics found in our CVID group are comparable to those reported previously in the literature: increased naïve and transitional B cells, CD21^{low} B cells and cTfh cells, and decreased memory B-cell subsets, total CD4$^+$ T-cell counts, naïve CD4$^+$ T cells and CD4$^+$ RTE;[24-30] heterogeneous distribution of peripheral B-cell patterns;[9,17,31] decreased proportions of NK cells and dendritic cells;[32,33] lower ICOS upregulation on activated T cells;[34] increased HLA-DR expression on B and T cells;[35,36] decreased BAFF-R, CXCR5 and CCR7 expression on B cells;[37,38] and decreased CCR7 expression on T cells, especially on cTfh cells.[38,39] It should be noted that the observed reduction of BAFF-R in CVID might be an overestimation because BAFF-R expression levels are lower on CD27$^-$ B cells, the predominant B-cell subset in these patients.

While CVID has been studied in depth over the past four decades, there are few immunophenotypic reports on milder forms of PAD, such as IPH and IgGSD. Our findings in IPH and IgGSD patients are compatible with previous published information on milder forms of PAD.[9,10,40,41] IPH and IgGSD patients showed an abnormal distribution of naïve and memory B- and T-cell subsets, similar to that observed in CVID patients, albeit to a lesser extent with a

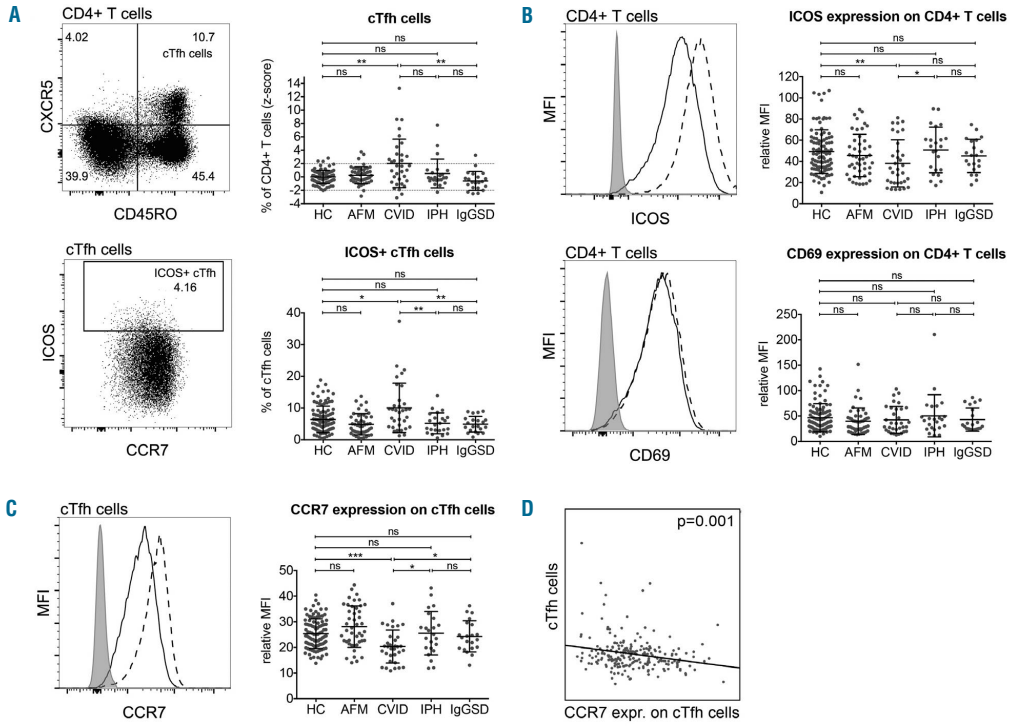


Figure 5. Circulating follicular helper T (cTfh) cells and expression of ICOS and CCR7 on cTfh cells. (A) Circulating follicular helper T (cTfh) cells were gated as CXCR5$^+$CD45RO$^+$ in CD4$^+$ T cells. ICOS$^+$ cTfh cells were gated based on "fluorescence minus one" (FMO). A representative gating strategy is shown on the left. Graphs represent mean ± SD. cTfh cells were expressed as z-scores to adjust for age-related differences in memory cells. Values normal for age have a z-score between -2 and 2 (dotted lines). (B) ICOS and CD69 expression on CD4$^+$ T cells stimulated with phytohemagglutinin for 72 h. CD69 was used as a positive control for T-cell activation. Graphs represent mean ± SD. Representative flow cytometric analysis is shown on the left. Full black lines represent CVID patients, dashed black lines represent HC. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population (black line) by the MFI of the FMO population (gray). (C) CCR7 expression on cTfh cells. The graph represents mean ± SD. Representative flow cytometric analysis is shown on the left. The full black line represents a CVID patient, the dashed black line represents a HC. Relative mean MFI was calculated by dividing the MFI of the positive population (black line) by the MFI of the FMO population (gray). AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia. *P≤0.05, **P≤0.01, ***P≤0.001, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons). (D) Correlation between CCR7 expression on cTfh cells and level of cTfh cells (Spearman rank correlation). Expr: expression.

block later in B-cell development and a less pronounced T-cell skewing towards memory subsets. Furthermore, compared to the CVID group, IPH and IgGSD patients had less severely decreased serum levels of all Ig isotypes. Like the CVID group, the IPH and IgGSD groups in our cohort had significantly reduced proportions of NK cells and dendritic cells. Moreover, they had a similar though less pronounced decrease in BAFF-R and increase in HLA-DR and CD40 expression on B cells. In contrast to CVID patients, IPH and IgGSD patients did not have significant alterations in mean cTfh cell levels, in mean chemokine receptor expression on B and T cells, or in mean ICOS upregulation on activated T cells. To our knowledge, this is the first report on co-stimulatory molecules and chemokine receptors on B and T cells in milder forms of PAD. While the immunophenotypic parameters of CVID, IPH and IgGSD patients appear to form a continuous spectrum, we observed small differences in the type of infections they developed. In particular, compared to CVID and IgGSD patients, a higher proportion of IPH patients had unusual infections (i.e. warts, fungal infections), although the difference was not statistically significant.

There have been no detailed immunophenotypic studies in cohorts of asymptomatic relatives of patients with PAD. Two previous studies investigated serum Ig levels in first-degree relatives of Iranian (n=64) and Turkish (n=63) CVID patients.[42,43] The proportion of asymptomatic relatives with reduced total IgG, IgM and/or IgA in the Iranian (13%) and Turkish (19%) cohorts was comparable to that in our cohort (8/47, 17%).[42,43] In our cohort, mean IgG and IgM levels were significantly lower in AFM than in HC suggesting that AFM are genetically and/or environmentally predisposed to the development of PAD. This hypothesis is further supported by the fact that several flow cytometric abnormalities detected in PAD patients were also found in AFM (Figure 7). Especially, AFM showed a similar trend regarding the profile of naïve and memory B and T cells, with significantly increased transitional B cells, $CD4^+$ TCM cells and $CD8^+$ TCM cells and significantly decreased IgD^-CD27^+ memory B cells, plasmablasts and $CD4^+$ RTE compared to HC. Moreover, the distribution of peripheral B-cell patterns among AFM was similar to that among IPH and IgGSD patients and indicated that some AFM have a defect in later stages of

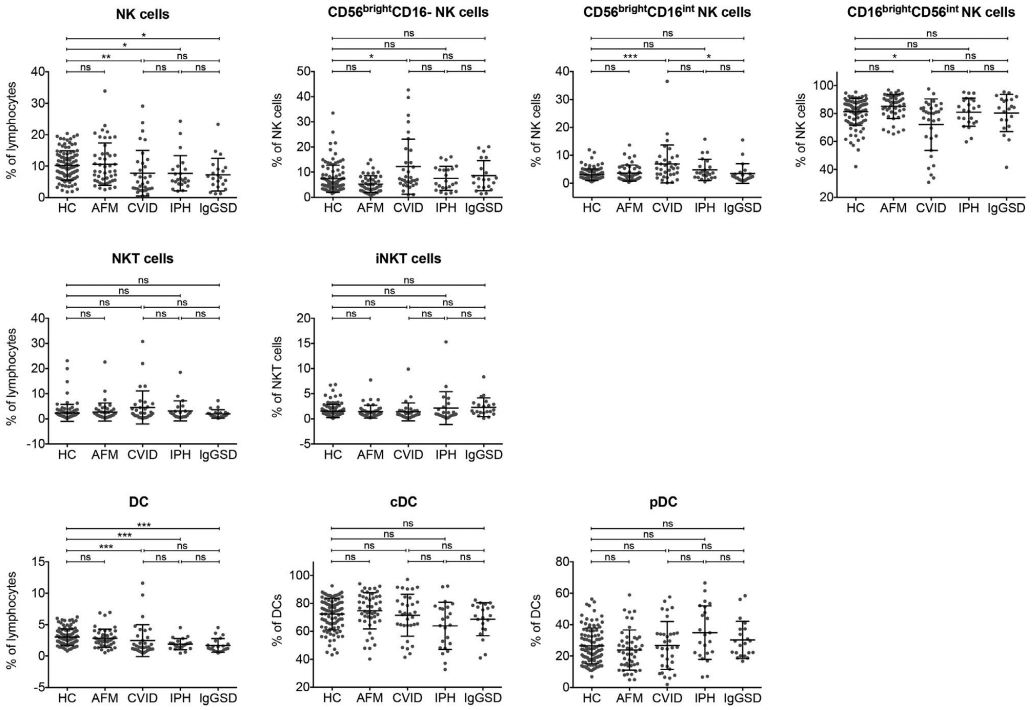


Figure 6. Innate immune cells: natural killer cells, natural killer T cells, dendritic cells and subsets. Natural killer (NK) cells were gated as $CD3^-CD56^+$ in $CD19^-HLA\text{-}DR^-$ lymphocytes. NK-cell subsets were determined by their relative expression of CD56 and CD16. Natural killer T (NKT) cells were gated as $CD3^+CD56^+$ in $CD19^-HLA\text{-}DR^-$ lymphocytes, and invariant NKT (iNKT) cells as invariant TCR (TCR Vα24-Jα18) positive in NKT cells. Dendritic cells (DC) were gated as lineage$^-CD4^+HLA\text{-}DR^+$ cells. Within DC, conventional DC (cDC) were gated as $CD11c^+CD123^-$ and plasmacytoid DC (pDC) as $CD123^+CD11c^-$. Graphs represent mean ± SD. AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia. *P≤0.05, **P≤0.01, ***P≤0.001, ns not significant (Mann-Whitney test with the Bonferroni correction for multiple comparisons).

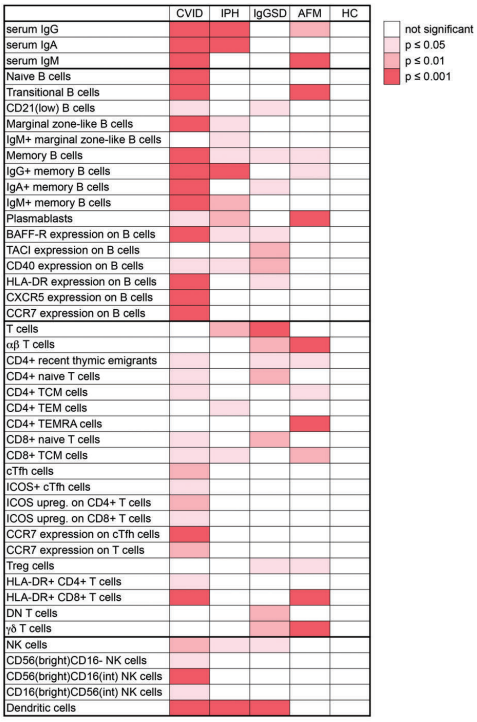


Figure 7. Summary of significant differences in the study. All parameters that tested significantly different in the study groups compared to HC are shown in a heat map with colours representing the corresponding *P*-values as indicated. AFM: asymptomatic family member; CVID: common variable immunodeficiency; HC: healthy control; IgGSD: IgG subclass deficiency; IPH: idiopathic primary hypogammaglobulinemia.

peripheral B-cell development. As opposed to B- and T-cell naïve and memory subsets, mean expression levels of the here-examined co-stimulatory molecules and chemokine receptors on B and T cells were not significantly altered in AFM compared to HC. Furthermore, AFM had, on average, normal levels of cTfh cells, NK cells, and dendritic cells (total and subsets). Together, our data suggest that a broader impairment of the immune system, including alterations in co-stimulatory molecules, lymphocyte trafficking and innate immune cells, is required to develop a clinical phenotype. It would be interesting to follow IPH and IgGSD patients as well as AFM over time (especially young individuals), to see whether their immunophenotype approaches that of patients with CVID. It should be noted that early Ig replacement therapy might interfere with the natural progression of the disease.

Several classifications have been proposed to distinguish subgroups among CVID patients, mainly using peripheral B- and/or T-cell immunophenotyping.[24-27,44,45] However, the plethora of immunological abnormalities and their unequal distribution among different cohorts have made it challenging to uniformly identify subgroups in the CVID population.[10,46-48] In our cohort, unsupervised computational clustering techniques were unable to reveal subgroups of subjects using different combinations of immunological and/or clinical parameters. The inability to distinguish clusters might be due to the relatively small groups. More probably, however, this reflects the heterogeneous nature of CVID and related PAD, and is in line with the fact that mild immunophenotypic abnormalities are already present in AFM. Possibly, genetic and/or environmental elements predisposing to the development of PAD may be more prevalent in the general community than initially thought. Depending on the degree to which these elements congregate, a wide range of phenotypes arises.

While we recognize that the small sample size of our study means that the findings warrant confirmation in larger cohorts, we conclude that the immunophenotypic landscape in CVID and other PAD comprises a varied and overlapping spectrum in which asymptomatic relatives show an intermediate immunophenotype, supporting the notion of a complex etiological basis of PAD.

Acknowledgments

The authors would like to thank the patients and their families and the healthy blood donors for participating in the study. We also thank the nurses for their assistance in blood sampling and Kelly Heyns and Nancy De Cabooter for their help with isolating peripheral blood mononuclear cells from blood samples. Finally, we are deeply grateful to Prof. Dr. Elfride De Baere for her constructive discussions.

References

1. Bruton OC. Agammaglobulinemia. Pediatrics. 1952;9(6):722-728.
2. Durandy A, Kracker S, Fischer A. Primary antibody deficiencies. Nat Rev Immunol. 2013;13(7):519-533.
3. Bonilla FA, Barlan I, Chapel H, et al. International Consensus Document (ICON): common variable immunodeficiency disorders. J Allergy Clin Immunol Pract. 2016;4(1):38-59.
4. Fudenberg H, Good RA, Goodman HC, et al. Primary immunodeficiencies. Report of a World Health Organization Committee. Pediatrics. 1971;47(5):927-946.
5. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol. 1999; 93(3):190-197.
6. Ameratunga R, Woon ST, Gillis D, Koopmans W, Steele R. New diagnostic criteria for common variable immune deficiency (CVID), which may assist with decisions to treat with intravenous or subcutaneous immunoglobulin. Clin Exp Immunol. 2013;174(2):203-211.
7. Ameratunga R, Brewerton M, Slade C, et al. Comparison of diagnostic criteria for common variable immunodeficiency disorder. Front Immunol. 2014;5:415.
8. Gathmann B, Mahlaoui N, Gerard L, et al. Clinical picture and treatment of 2212 patients with common variable immunodeficiency. J Allergy Clin Immunol. 2014;134(1):116-126.
9. Driessen GJ, Dalm VA, van Hagen PM, et al. Common variable immunodeficiency and idiopathic primary hypogammaglobulinemia: two different conditions within the same disease spectrum. Haematologica. 2013;98(10):1617-1623.
10. van de Ven AA, van Montfrans JM. Clinical complications in pediatric CVID are not restricted to patients with severely reduced class-switched memory B cells. Pediatr Allergy Immunol. 2011;22(3):347-348.
11. Kutukculer N, Gulez N. The outcome of patients with unclassified hypogammaglobulinemia in early childhood. Pediatr Allergy

Immunol. 2009;20(7):693-698.
12. Pan Q, Hammarstrom L. Molecular basis of IgG subclass deficiency. Immunol Rev. 2000;178:99-110.
13. Fried AJ, Bonilla FA. Pathogenesis, diagnosis, and management of primary antibody deficiencies and infections. Clin Microbiol Rev. 2009;22(3):396-414.
14. Bogaert DJA, Dullaers M, Lambrecht BN, Vermaelen KY, De Baere E, Haerynck F. Genes associated with common variable immunodeficiency: one diagnosis to rule them all? J Med Genet. 2016;53(9):575-90.
15. van Schouwenburg PA, Davenport EE, Kienzler AK, et al. Application of whole genome and RNA sequencing to investigate the genomic landscape of common variable immunodeficiency disorders. Clin Immunol. 2015;160(2):301-314.
16. Rodriguez-Cortez VC, Del Pino-Molina L, Rodriguez-Ubreva J, et al. Monozygotic twins discordant for common variable immunodeficiency reveal impaired DNA demethylation during naive-to-memory B-cell transition. Nat Commun. 2015;6:7335.
17. Driessen GJ, van Zelm MC, van Hagen PM, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood. 2011;118(26): 6814-6823.
18. Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol. 2014;32: 659-702.
19. Liu X, Nurieva RI, Dong C. Transcriptional regulation of follicular T-helper (Tfh) cells. Immunol Rev. 2013;252(1):139-145.
20. Fliegauf M, Bryant VL, Frede N, et al. Haploinsufficiency of the NF-kappaB1 subunit p50 in common variable immunodeficiency. Am J Hum Genet. 2015;97(3):389-403.
21. Kuehn HS, Ouyang W, Lo B, et al. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. Science. 2014;345(6204):1623-1627.
22. Schubert D, Bode C, Kenefeck R, et al. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. Nat Med. 2014;20(12):1410-1416.
23. Kuehn HS, Boisson B, Cunningham-Rundles C, et al. Loss of B cells in patients with heterozygous mutations in IKAROS. N Engl J Med. 2016;374(11):1032-1043.
24. Warnatz K, Denz A, Drager R, et al. Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood. 2002;99(5): 1544-1551.
25. Piqueras B, Lavenu-Bombled C, Galicier L, et al. Common variable immunodeficiency patient classification based on impaired B cell memory differentiation correlates with clinical aspects. J Clin Immunol. 2003;23(5): 385-400.
26. Wehr C, Kivioja T, Schmitt C, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood. 2008;111(1):77-85.
27. Giovannetti A, Pierdominici M, Mazzetta F, et al. Unravelling the complexity of T cell abnormalities in common variable immunodeficiency. J Immunol. 2007;178(6):3932-3943.
28. Romberg ND, Hsu I, Price CC, Cunningham-Rundles C, Meffre E. Expansion of circulating T follicular helper cells in CVID patients with autoimmune cytopenias. J Allergy Clin Immunol. 2014;133(2):AB162-AB162.
29. Malphettes M, Gerard L, Carmagnat M, et al. Late-onset combined immune deficiency: a subset of common variable immunodeficiency with severe T cell defect. Clin Infect Dis. 2009;49(9):1329-1338.
30. De Vera MJ, Al-Harthi L, Gewurz AT. Assessing thymopoiesis in patients with common variable immunodeficiency as measured by T-cell receptor excision circles. Ann Allergy Asthma Immunol. 2004;93(5): 478-484.
31. Piatosa B, Pac M, Siewiera K, et al. Common variable immune deficiency in children-clinical characteristics varies depending on defect in peripheral B cell maturation. J Clin Immunol. 2013;33(4): 731-741.
32. Aspalter RM, Sewell WA, Dolman K, Farrant J, Webster AD. Deficiency in circulating natural killer (NK) cell subsets in common variable immunodeficiency and X-linked agammaglobulinaemia. Clin Exp Immunol. 2000;121 (3):506-514.
33. Cunningham-Rundles C, Radigan L. Deficient IL-12 and dendritic cell function in common variable immune deficiency. Clin Immunol. 2005;115(2):147-153.
34. Berron-Ruiz L, Lopez-Herrera G, Vargas-Hernandez A, et al. Lymphocytes and B-cell abnormalities in patients with common variable immunodeficiency (CVID). Allergol Immunopathol. 2014;42(1):35-43.
35. Viallard JF, Blanco P, Andre M, et al. CD8+HLA-DR+ T lymphocytes are increased in common variable immunodeficiency patients with impaired memory B-cell differentiation. Clin Immunol. 2006;119(1): 51-58.
36. Carter CR, Aravind G, Smalle NL, Cole JY, Savic S, Wood PM. CVID patients with autoimmunity have elevated T cell expression of granzyme B and HLA-DR and reduced levels of Treg cells. J Clin Pathol. 2013;66(2):146-150.
37. Barbosa RR, Silva SL, Silva SP, et al. Reduced BAFF-R and increased TACI expression in common variable immunodeficiency. J Clin Immunol. 2014;34(5):573-583.
38. Payne D, Drinkwater S, Baretto R, Duddridge M, Browning MJ. Expression of chemokine receptors CXCR4, CXCR5 and CCR7 on B and T lymphocytes from patients with primary antibody deficiency. Clin Exp Immunol. 2009;156(2):254-562.
39. Holm AM, Sivertsen EA, Tunheim SH, et al. Gene expression analysis of peripheral T cells in a subgroup of common variable immunodeficiency shows predominance of CCR7(-) effector-memory T cells. Clin Exp Immunol. 2004;138(2):278-289.
40. van de Ven AA, van de Corput L, van Tilburg CM, et al. Lymphocyte characteristics in children with common variable immunodeficiency. Clin Immunol. 2010; 135(1):63-71.
41. Barton JC, Bertoli LF, Barton JC. Comparisons of CVID and IgGSD: referring physicians, autoimmune conditions, pneumovax reactivity, immunoglobulin levels, blood lymphocyte subsets, and HLA-A and -B typing in 432 adult index patients. J Immunol Res. 2014;2014:542706.
42. Aghamohammadi A, Sedighipour L, Saeed SE, Kouhkan A, Heydarzadeh M, Pourpak Z. Alterations in humoral immunity in relatives of patients with common variable immunodeficiency. J Investig Allergol Clin Immunol. 2008;18(4):266-271.
43. Karakoc-Aydiner E, Ozen AO, Baris S, Ercan H, Ozdemir C, Barlan IB. Alteration in humoral immunity is common among family members of patients with common variable immunodeficiency. J Investig Allergol Clin Immunol. 2014;24(5):346-351.
44. Picat M-Q, Thiebaut R, Lifermann F, et al. T-cell activation discriminates subclasses of symptomatic primary humoral immunodeficiency diseases in adults. BMC Immunol. 2014;15:13.
45. Rosel AL, Scheibenbogen C, Schliesser U, et al. Classification of common variable immunodeficiencies using flow cytometry and a memory B-cell functionality assay. J Allergy Clin Immunol. 2015;135(1):198-208.
46. Kutukculer N, Gulez N, Karaca NE, Aksu G, Berdeli A. Three different classifications, B lymphocyte subpopulations, TNFRSF13B (TACI), TNFRSF13C (BAFF-R), TNFSF13 (APRIL) gene mutations, CTLA-4 and ICOS gene polymorphisms in Turkish patients with common variable immunodeficiency. J Clin Immunol. 2012;32(6):1165-1179.
47. Koopmans W, Woon ST, Zeng IS, Jordan A, Brothers S, Browett P, et al. Variability of memory B cell markers in a cohort of common variable immune deficiency patients over 6 months. Scand J Immunol. 2013;77(6): 470-475.
48. Al Kindi M, Mundy J, Sullivan T, et al. Utility of peripheral blood B cell subsets analysis in common variable immunodeficiency. Clin Exp Immunol. 2012;167 (2):275-281.

Supplementary Information

SUPPLEMENTARY METHODS

Serum immunoglobulin levels

Serum samples of AFM and HC were cryopreserved at -80°C and immunoglobulin (Ig)G, IgG2, IgG3, IgA and IgM concentrations were measured on thawed serum by nephelometry (Behring Nephelometer Analyzer II). Ig levels of patients had been previously determined at time of diagnosis, on fresh serum samples using nephelometry (Behring Nephelometer Analyzer II).

Absolute white blood cell counts

Absolute white blood cell counts and differentiations were determined on EDTA whole blood of patients during routine lab evaluations by means of a Sysmex XE-5000 (Sysmex), within a six-month range around time of inclusion in the study. On the same sample, B, T and NK cells were measured using a BD FACSCanto II flow cytometer (BD Biosciences) and FACSDiva software version 8 (BD Biosciences).
Due to practical reasons, absolute white blood cell counts on EDTA whole blood could not be assessed in asymptomatic family members (AFM) or healthy controls (HC).

Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from EDTA whole blood by Ficoll-Paque density gradient centrifugation and cryopreserved at -150°C for batch analysis. Thawed PBMCs were stained with fixable viability dye 506 (eBioscience) and fluorescently labeled monoclonal antibodies under saturation conditions. Following monoclonal antibodies were used (clones specified between brackets): CD8 (RPA-T8), CD14 (MΦP9), CD16 (3G8), CD19 (HIB19), CD20 (2H7), CD21 (B-LY4), CD27 (M-T271), CD31 (WM59), CD40 (5C3), CD138 (MI15), CXCR5 (RF8B2), IgD (IA6-2), IgM (G20-127), γδTCR (11F2) (all BD Biosciences); CD3 (SK7), CD4 (SK3), CD11c (BU15), CD19 (HIB19), CD20 (2H7), CD24 (ML5), CD25 (BC96), CD34 (581), CD45RO (UCHL1), CD123 (6H6), CD197(CCR7) (G043H7), CD267(TACI) (1A1), CD268(BAFF-R) (11C1), CD278(ICOS) (C398.4A), HLA-DR (L243) (all Biolegend); CD4 (RPA-T4), CD38 (HIT2), CD56 (TULY56), Foxp3 (PCH101) (all eBioscience); IgA (IS11-8E10), IgG (IS11-3B2.2.3), iNKT (6B11) (all Miltenyi Biotec).
To analyze ICOS upregulation on T cells, thawed PBMCs were incubated with 1% PHA (Life Technologies) for 72 hours at 37°C in 5% CO_2 at a density of 1.25 x 10^6 PBMCs/mL in

supplemented RPMI medium (Gibco). Afterwards, cells were stained with fixable viability dye 506 (eBioscience) and fluorescently labeled monoclonal antibodies under saturation conditions: CD69-BV605 (FN50) (BD Biosciences); CD3-PerCP-Cy5.5 (SK7), CD4-Pacific Blue (SK3), CD278(ICOS)-FITC (C398.4A) (all Biolegend); CD8-APC (BW135/80) (Miltenyi Biotec). CD69 expression was used as a positive control for T cell activation.
Cells were acquired on an LSR Fortessa flow cytometer with 3 lasers (488nm blue laser, 405 nm violet laser, 640nm red laser) that can measure up to 12 colors simultaneously (BD Biosciences). At least 100 000 events per sample were recorded. Data were analyzed with FlowJo version X (Tree Star Inc.).

Unsupervised computational clustering analysis

Unsupervised clustering analysis was performed on log2 transformed serum Ig levels and flow cytometry variables using the R programming language (v3.1.1) (1). The immunological data was studied for sample subgroups by means of hierarchical clustering – implementing the "manhattan" distance method and the "ward.D2" clustering method – through the R package "pheatmap" (v1.0.7) (2) to provide the accompanying heatmaps. Additionally, principal component analysis (PCA) was performed using the base R function "prcomp()". Both clustering methods were evaluated on data from (i) all samples included in the study, (ii) patients and AFM, (iii) patients only and (iv) each individual PAD entity.

Statistics

Statistical analysis was performed with SPSS Statistics version 22 (IBM®). Variables were not normally distributed; therefore non-parametrical statistical tests were used throughout. Continuous variables between multiple groups were compared using the Kruskal-Wallis test. If the former indicated significant differences, pairwise comparison of groups was done using the Mann-Whitney test with Bonferroni's post hoc correction for multiple comparisons. Categorical variables between multiple groups were compared using the chi square test. If this indicated significant differences, pairwise comparison of groups was done using the Fisher's Exact test with Bonferroni's post hoc correction for multiple comparisons. Correlations were calculated with Spearman Rank Correlation. A two-sided p value ≤ 0.05 was considered statistically significant.
Continuous variables were converted into z-scores to adjust for age-dependent differences when required. A z-score is the number of standard deviations (SD) the measured value is above or below the normal mean for age: $z = \frac{x - \mu}{\sigma}$, x being the measured value, μ the mean value of the age-based reference population, and σ the SD of the age-based

reference population (3). Values normal for age have a z-score between -2 and +2 (i.e. 2 SD below and above the age-adjusted mean). Z-scores of Ig levels and absolute white blood cell counts were calculated using age-based reference values from the routine lab. Z-scores of naive and memory PBMC subsets were calculated using age-based reference values derived from the HC group. The division of the healthy controls into age groups and the corresponding reference values are given in Table S1.

SUPPLEMENTARY RESULTS

Characteristics of the study population: absolute white blood cell counts.

Absolute white blood cell counts were only determined in patients during routine lab evaluations. These routine lab evaluations were done within a six-month range around time of analysis for the study.

Mean lymphocyte, B cell, and $CD4^+$ T cell counts were significantly lower in CVID compared to IPH and IgGSD (Figure S1). At individual level, lymphopenia was observed in six of 33 CVID patients (Table S2). Eleven CVID patients and one IgGSD patient had reduced B cell counts (Table S2). Total absolute T cell counts were mildly decreased in five CVID patients of whom two had decreased $CD4^+$ T cells, one decreased $CD8^+$ T cell numbers and two decreased $CD4^+$ and $CD8^+$ T cell numbers (Table S2). Note that for B, T, $CD4^+$ T, $CD8^+$ T, and NK cells, absolute counts measured in the routine lab were strongly correlated with percentages determined on flow cytometry (all $p<0.001$; data not shown).

Associations between clinical features and the immunophenotype in patients.

Flow cytometric B and/or T cell phenotyping is frequently used to discriminate CVID patients at risk for non-infectious complications (4, 5). Therefore, associations between clinical features and immunological parameters were examined in all PAD patients together and in CVID, IPH and IgGSD patients separately. Statistical data on the here-discussed associations are provided in Table S4.

In our cohort, PAD patients with chronic lung disease (i.e. bronchiectasis, lung granulomata and/or lymphocytic interstitial pneumonitis; n=22) (Table S3) had significantly increased $CD21^{low}$ B cells as well as significantly decreased IgG, IgM, IgA, absolute B cell numbers and IgD^-CD27^+ memory B cells compared to PAD patients without chronic lung disease. Within the separate groups of CVID, IPH and IgGSD patients, we could not detect a significant link between chronic lung disease and any of the immunological parameters, which could be due to the small group sizes.

Polyclonal lymphoproliferative disease was defined as the presence of benign lymphadenopathy (cervical, mediastinal and/or abdominal lymph nodes > 1 cm diameter, detected at least twice on medical imaging), hepatomegaly (as protocolled upon abdominal ultrasound), and/or splenomegaly (as protocolled upon abdominal ultrasound) (Table S3). PAD patients with polyclonal lymphoproliferative disease (n=17) had significantly higher naive B cells, $CD21^{low}$ B cells, $CD4^+$ TCM cells and cTfh cells compared to those without. Furthermore, PAD patients with lymphoproliferative disease had significantly lower IgG, IgM, IgA, IgD^-CD27^+ memory B cells, naive CD4+ T cells, $CD4^+$ RTE, naive $CD8^+$ T cells, CXCR5 and CCR7 expression on B cells, and CCR7 expression on cTfh cells. Within CVID patients

separately, those with polyclonal lymphoproliferation (n=14) were found to have significantly increased $CD4^+$ TCM cells and cTfh cells and significantly decreased IgG, naive $CD4^+$ T cells, $IgD^-CD27^+IgA^+$ memory B cells, and CXCR5 expression on B cells compared to those without lymphoproliferative disease. There were no immunological differences between IPH patients with and without polyclonal lymphoproliferative disease. None of the IgGSD patients had developed lymphoproliferative disease at time of analysis.

PAD patients with autoimmunity (i.e. symptoms related to autoimmune disease; n=19) (Table S3) had significantly increased $CD4^+$ TCM cells and cTfh cells and significantly decreased IgA, naive $CD4^+$ T cells and $CD4^+$ RTE. PAD patients with autoimmunity also showed a trend towards increased TACI expression on B cells but this did not reach statistical significance. However, in PAD patients with autoimmune cytopenia (n=6), TACI expression was significantly higher compared to those without autoimmune cytopenia. Within CVID patients separately, those with autoimmunity (n=13) showed significantly higher $CD4^+$ TCM cells and TACI expression on B cells compared to those without autoimmune manifestations. The increased TACI expression on B cells was even more significant in CVID patients with autoimmune cytopenias (n=5) compared to those without. IPH patients with autoimmunity (n=3) also demonstrated a non-statistically significant trend towards higher $CD4^+$ TCM cells compared to those without autoimmunity. IgGSD patients with and without autoimmunity did not have significantly different immunological parameters.

SUPPLEMENTARY TABLES

Table S1. Age-based reference values derived from the healthy control group.

Age group (years)	N	Naive B cells (% B cells)	Transitional B cells (% B cells)	$CD21^{low}$ B cells (% B cells)	$IgD^{-}CD27^{+}$ memory B cells (% B cells)	$IgD^{+}CD27^{+}$ marginal zone-like B cells (% B cells)	Plasmablasts (% B cells)	Plasma cells (% alive)
4.5 - 10.0	11	54.80 - 73.72	1.80 - 6.17	0.00 - 10.75	11.31 - 24.09	3.04 - 15.23	0.04 - 1.85	0.20 - 0.61
10.1 - 16.0	15	45.55 - 92.68	0.06 - 13.67	0.60 - 3.34	1.81 - 29.95	0.00 - 19.32	0.00 - 2.66	0.00 - 1.09
16.1 - 20.0	10	57.64 - 83.58	0.20 - 5.82	0.12 - 3.50	6.37 - 25.71	1.49 - 12.31	0.14 - 1.57	0.08 - 0.91
20.1 - 30.0	21	40.26 - 87.39	0.00 - 6.20	0.00 - 5.65	3.05 - 37.64	0.61 - 15.91	0.10 - 2.02	0.00 - 1.99
30.1 - 40.0	14	25.81 - 78.79	0.23 - 4.88	0.22 - 7.25	5.81 - 52.36	2.15 - 21.56	0.52 - 1.47	0.12 - 1.30
40.1 - 50.0	12	25.26 - 88.85	0.00 - 4.06	0.00 - 6.96	2.84 - 52.59	0.60 - 17.51	0.00 - 1.88	0.00 - 1.68
50.1 - 90.0	18	0.00 - 82.40	1.18 - 6.60	7.56 - 15.93	17.64 - 48.17	8.51 - 25.31	0.97 - 1.68	0.81 - 2.25

Age group (years)	N	Naive $CD4^{+}$ T cells (% $CD4^{+}$ T cells)	$CD4^{+}$ RTE (% $CD4^{+}$ T cells)	$CD4^{+}$ TCM (% $CD4^{+}$ T cells)	$CD4^{+}$ TEM (% $CD4^{+}$ T cells)	$CD4^{+}$ TEMRA (% $CD4^{+}$ T cells)	Naive $CD8^{+}$ T cells (% $CD8^{+}$ T cells)	$CD8^{+}$ TCM (% $CD8^{+}$ T cells)	$CD8^{+}$ TEM (% $CD8^{+}$ T cells)	$CD8^{+}$ TEMRA (% $CD8^{+}$ T cells)	cTfh cells (% $CD4^{+}$ T cells)
4.5 - 10.0	11	47.61 - 79.07	38.81 - 66.77	15.13 - 30.42	3.29 - 22.40	0.00 - 2.20	36.15 - 81.45	0.00 - 10.29	8.55 - 34.48	0.00 - 30.76	5.04 - 12.46
10.1 - 16.0	15	42.54 - 82.41	28.47 - 64.34	9.15 - 34.41	3.64 - 25.02	0.00 - 5.02	29.80 - 96.09	1.18 - 9.36	0.00 - 39.58	0.00 - 29.57	1.97 - 11.06
16.1 - 20.0	10	45.55 - 68.95	31.99 - 57.49	16.00 - 34.67	4.57 - 28.25	0.00 - 2.45	37.78 - 84.72	1.27 - 8.14	9.23 - 33.72	0.00 - 29.50	3.44 - 12.10
20.1 - 30.0	21	35.32 - 74.69	20.09 - 58.55	14.88 - 46.60	3.19 - 23.65	0.00 - 2.05	28.27 - 82.58	2.67 - 13.31	7.93 - 43.52	0.00 - 22.30	4.49 - 14.64
30.1 - 40.0	14	15.67 - 63.65	7.10 - 44.73	23.82 - 57.75	3.29 - 33.55	0.00 - 2.85	10.40 - 69.52	5.43 - 16.97	8.60 - 56.61	0.00 - 39.99	4.86 - 23.45
40.1 - 50.0	12	25.82 - 70.36	10.25 - 47.73	19.45 - 56.57	4.98 - 19.58	0.00 - 4.39	7.73 - 61.28	3.99 - 20.98	6.78 - 61.64	0.00 - 46.61	2.09 - 21.11
50.1 - 90.0	18	0.00 - 66.33	0.00 - 43.45	21.88 - 57.36	13.54 - 37.18	3.32 - 11.89	0.00 - 54.35	14.42 - 30.61	23.53 - 55.37	30.98 - 68.78	5.51 - 15.16

Healthy controls (HC) were divided into seven age groups. Reference values were calculated as mean ± two times the standard deviation of the corresponding age group. Negative values for the lower limit were set at 0.00. cTfh: circulating follicular helper T, N: number of HC in age group, RTE: recent thymic emigrants, TCM: central memory T, TEM: effector memory T, TEMRA: effector memory RA T.

Table S2. White blood cell counts of the patients.

Patient	Diagno-sis	Gender	Age (y)	Neutro (/µL)	Mono (/µL)	Lympho (/µL)	T cells (/µL)	CD4+ T (/µL)	CD8+ T (/µL)	B cells (/µL)	NK cells (/µL)
P1	CVID	Male	61.9	6690	690	2010	1670	362	1250	121	221
P2	CVID	Male	14.3	2470*	700	1700	1260	663	442	255	153
P3	CVID	Female	17.0	3860	550	2060	1774	1296	476	99	46*
P4	CVID	Male	13.6	5290	910	1190*	940	643	274	36*	179
P5	CVID	Female	10.8	6080	960	2410	1740	1080	603	48*	554
P6	CVID	Female	14.1	1870*	260*	1580	1200	664	427	253	126
P7	CVID	Female	49.0	4070	362	1538	1170	769	369	246	108
P8	CVID	Male	14.6	1490*	350*	580*	447*	290*	122*	87*	23.2*
P9	CVID	Male	18.2	2230	420	3610	2100	1360	679	1050	170
P10	CVID	Male	7.8	6980	690*	3990	2630	1600	838	798	479
P11	CVID	Male	7.8	3150	570*	3480	2580	1640	800	592	244
P12	CVID	Male	7.7	1550*	560*	1430	849	449	349	200	175
P13	CVID	Female	24.7	2340	330	1380	1310	804	459	115	180
P14	CVID	Female	14.0	4060	980	2350	1370	659	575	17*	304
P15	CVID	Female	9.4	3280	470*	2150	1700	862	583	117*	489
P16	CVID	Female	33.1	2601	213*	1160	1170	471	638	152	197
P17	CVID	Female	23.4	10400	2000	2580	2000	942	667	184	115
P18	CVID	Male	71.8	3368	590	868*	590*	174*	434	0*	234
P19	CVID	Male	43.7	5050	510	860*	559*	430	112*	155	120
P20	CVID	Female	83.2	4900	740	3020	2473	1389	1084	255	130
P21	CVID	Male	16.6	2780	530	1880	2270	1010	982	706	92.1
P22	CVID	Female	28.8	2884	831	4642	2880	1440	1390	1440	232
P23	CVID	Male	17.3	3090	760	2030	1400	974	365	386	223
P24	CVID	Male	16.6	2750	420*	2200	1780	781	805	317	293
P25	CVID	Male	32.3	3230	430	2480	1440	521	843	50*	918
P26	CVID	Female	45.0	3865	475	1966	1320	923	308	325	68.4*
P27	CVID	Male	53.6	6350	630	1370	740	480	219	41*	534
P28	CVID	Male	11.6	6210	1400	1680	1100	389	443	142*	478
P29	CVID	Male	13.8	780*	300*	1300*	552*	256*	280	200	32*
P30	CVID	Male	17.8	4000	580	1640	935	476	394	246	426
P31	CVID	Male	80.8	5890	680	1280	1210	604	574	30*	227
P32	CVID	Female	36.7	3800	390	1870	1570	617	879	243	37.4*
P33	CVID	Female	14.6	1130*	360*	650*	496*	306*	132*	174*	149
P34	IPH	Female	20.2	2200	320	1410	1640	1010	560	314	269
P35	IPH	Male	15.2	2860	260*	2080	2110	1300	572	312	156
P36	IPH	Female	22.6	1700	320	2210	2819	1879	766	487	139
P37	IPH	Female	37.4	7380	690	2070	1770	1230	517	492	172
P38	IPH	Female	37.5	3555	289	1879	939	543	352	132	358
P39	IPH	Female	30.5	4030	390	2010	1880	1010	746	265	241
P40	IPH	Female	68.6	4590	390	1240	982	742	241	149	75*
P41	IPH	Male	13.4	1890*	560	1950	1270	761	312	332	312
P42	IPH	Female	52.8	3520	440	1309	1140	940	198	379	115
P43	IPH	Male	14.2	3330	310*	1640	1000	623	279	476	131
P44	IPH	Female	50.9	4181	212*	2552	1910	1580	332	434	179
P45	IPH	Male	37.3	4600	730	2260	1780	725	681	220	198
P46	IPH	Male	86.5	2840	530	1570	1046	600	446	94	320
P47	IPH	Female	38.2	3929	315	2874	2302	1517	785	316	139
P48	IPH	Female	30.2	2440	350	1630	1320	848	424	147	163
P49	IPH	Male	50.1	2890	370	2770	2350	1270	942	249	139
P50	IPH	Male	11.9	1660*	320*	1810	1320	796	416	253	199
P51	IPH	Male	10.4	1800*	460*	2730	1100	713	356	300	146
P52	IPH	Female	81.6	8930	860	3930	3152	2727	424	150	393
P53	IPH	Female	28.7	3788	302	5490	877	525	352	99	58*
P54	IPH	Female	52.8	5770	1000	3020	2140	1540	604	513	302
P55	IPH	Male	10.3	3730	350*	2840	2240	909	1160	227	341
P56	IPH	Female	17.3	3010	300*	3010	2468	1355	903	361	151
P57	IgGSD	Female	49.8	4130	580	2600	2132	1482	624	182	260
P58	IgGSD	Female	11.9	3550	460*	4040	2200	1170	722	361	452
P59	IgGSD	Female	21.5	3820	1080	4190	3430	2300	1090	609	304
P60	IgGSD	Female	34.1	2800	650	2700	1660	1100	430	301	172
P61	IgGSD	Female	46.4	3280	890	2920	2316	1559	756	225	208
P62	IgGSD	Female	35.2	7469	747	2241	2960	1810	1020	165	165
P63	IgGSD	Female	34.7	5790	930	3360	2130	1450	568	454	227
P64	IgGSD	Female	42.9	2270	500	1620	2070	1540	498	249	174
P65	IgGSD	Female	57.5	3070	550	2300	1750	1290	437	322	230
P66	IgGSD	Female	42.2	4720	490	1490	1200	909	271	232	483
P67	IgGSD	Male	40.6	2434	667	2240	2120	1290	647	441	323
P68	IgGSD	Female	51.3	2843	202*	1444	1140	881	260	188	87
P69	IgGSD	Female	65.0	6969	481	1362	1140	708	409	41*	163
P70	IgGSD	Female	51.3	4045	502	1772	1520	1050	472	328	185
P71	IgGSD	Female	31.5	7669	327	1374	1060	715	289	206	96
P72	IgGSD	Female	56.4	5766	255	1123	831	550	269	123	157
P73	IgGSD	Female	32.5	3930	550	1420	852	650	308	270	209
P74	IgGSD	Female	46.0	5070	300	1750	1420	910	490	140	175
P75	IgGSD	Female	35.1	6465	172*	1853	1480	982	463	204	167
P76	IgGSD	Male	17.9	3090	460*	1990	1510	856	537	179	279
P77	IgGSD	Male	15.3	1590*	320*	1900	1060	494	494	380	437

*Below age-based reference values. CVID: common variable immunodeficiency, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia, Lympho: lymphocytes, Mono: monocytes, Neutro: neutrophils.

Table S3. Clinical characteristics of the patients.

	CVID	IPH	IgGSD
Infections and infection-related complications			
Recurrent upper respiratory tract infections	32/33 (97.0%)	23/23 (100%)	21/21 (100%)
Recurrent and/or severe lower respiratory tract infections	25/33 (75.8%)	20/23 (87.0%)	13/21 (61.9%)
Recurrent gastrointestinal infections	6/33 (18.2%)	3/23 (13.0%)	1/21 (4.8%)
Bacterial skin infections	2/33 (6.1%)	3/23 (13.0%)	2/21 (9.5%)
Deep abscesses (organ/muscle)	2/33 (6.1%)	1/23 (4.3%)	0/21 (0%)
Recurrent or invasive herpes simplex infections	1/33 (3.0%)	1/23 (4.3%)	1/21 (4.8%)
Recurrent herpes zoster infections	2/33 (6.1%)	3/23 (13.0%)	0/21 (0%)
Recurrent warts	1/33 (3.0%)	2/23 (8.7%)	0/21 (0%)
Recurrent fungal infections	1/33 (3.0%)	4/23 (17.4%)	2/21 (9.5%)
Bronchiectasis on HRCT thorax	13/24* (54.2%)	4/13* (30.8%)	2/12* (16.7%)
Non-infectious complications			
Unexplained enteropathy	13/33 (39.4%)	7/23 (30.4%)	9/21 (42.9%)
Benign lymphadenopathy on medical imaging[$]	11/33 (33.3%)	3/23 (13.0%)	2/21 (9.5%)
Lymphocytic interstitial pneumonitis	2/33 (6.1%)	0/23 (0%)	0/21 (0%)
Granulomata on CT thorax and/or coloscopy	6/32* (18.8%)	0/23 (0%)	0/21 (0%)
Splenomegaly on abdominal ultrasound	8/28* (28.6%)	1/14* (7.1%)	0/10* (0%)
Splenectomy	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
Hepatomegaly on abdominal ultrasound	6/28* (21.4%)	2/14* (14.3%)	0/10* (0%)
Acute non-infectious hepatitis	2/33 (6.1%)	1/23 (4.3%)	1/21 (4.8%)
Liver transplantation	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
Solid organ tumor	3/33 (9.1%)	0/23 (0%)	1/21 (4.8%)
Autoimmune manifestations	13/33 (39.4%)	3/23 (13.0%)	3/21 (14.3%)
- Autoimmune cytopenia	5/33 (15.2%)	1/23 (4.3%)	0/21 (0%)
- Inflammatory bowel disease	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
- Autoimmune thyroid disease	1/33 (3.0%)	1/23 (4.3%)	1/21 (4.8%)
- Rheumatic disease (JIA, RA)	2/33 (6.1%)	0/23 (0%)	1/21 (4.8%)
- Pernicious anemia	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
- Raynaud phenomenon	1/33 (3.0%)	0/23 (0%)	0/21 (0%)
- Sicca syndrome	0/33 (0%)	1/23 (4.3%)	0/21 (0%)
- Alopecia	1/33 (3.0%)	1/23 (4.3%)	0/21 (0%)
- Vitiligo	1/33 (3.0%)	1/23 (4.3%)	0/21 (0%)
- Lichen ruber planus	0/33 (0%)	0/23 (0%)	1/21 (4.8%)
Other			
Growth delay	5/33 (15.2%)	1/23 (4.3%)	0/21 (0%)

*Missing data (no medical imaging performed). [$]Cervical, mediastinal and/or abdominal lymph nodes > 1 cm diameter, detected at least twice on medical imaging. CVID: common variable immunodeficiency, (HR)CT: (high-resolution) computed tomography, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia, JIA: juvenile idiopathic arthritis, RA: rheumatoid arthritis.

Table S4. Most important associations between with clinical features and immunological parameters in patients.

	PAD with chronic lung disease* (n=22)	**PAD without chronic lung disease* (n=27)**	**P value**
Serum IgG (z-score, mean ± SD)	-3.2 ± 1.1	-2.0 ± 1.6	0.005
Serum IgM (z-score, mean ± SD)	-2.2 ± 1.2	-1.0 ± 1.6	0.003
Serum IgA (z-score, mean ± SD)	-2.5 ± 0.6	-1.3 ± 1.7	0.004
Absolute B cell numbers (z-score, mean ± SD)	-1.4 ± 1.6	0.28 ± 2.1	0.021
IgD^-CD27^+ memory B cells (z-score, mean ± SD)	-1.7 ± 1.5	-0.63 ± 1.1	0.007
$CD21^{low}$ B cells (z-score, mean ± SD)	5.8 ± 11.7	1.5 ± 5.1	0.016
	PAD with polyclonal lymphoproliferation$ (n=17)	**PAD without polyclonal lymphoproliferation$ (n=60)**	**P value**
Serum IgG (z-score, mean ± SD)	-3.6 ± 0.82	-2.0 ± 1.4	<0.001
Serum IgM (z-score, mean ± SD)	-2.1 ± 0.75	-1.3 ± 1.4	0.003
Serum IgA (z-score, mean ± SD)	-2.8 ± 0.38	-1.5 ± 1.7	<0.001
Naive B cells (z-score, mean ± SD)	1.5 ± 1.3	0.53 ± 1.6	0.01
IgD^-CD27^+ memory B cells (z-score, mean ± SD)	-1.8 ± 1.1	-0.95 ± 1.4	0.005
$CD21^{low}$ B cells (z-score, mean ± SD)	6.6 ± 14.1	0.81 ± 3.7	0.037
Naive $CD4^+$ T cells (z-score, mean ± SD)	-1.6 ± 2.1	0.31 ± 1.5	<0.001
$CD4^+$ RTE (z- score, mean ± SD)	-1.18 ± 1.5	0.15 ± 1.3	0.005
$CD4^+$ TCM cells (z-score, mean ± SD)	1.6 ± 1.6	-0.07 ± 1.4	<0.001
cTfh cells (z-score, mean ± SD)	3.5 ± 3.7	0.12 ± 2.2	<0.001
Naive $CD8^+$ T cells (z-score, mean ± SD)	-0.61 ± 1.1	0.17 ± 1.2	0.019
CXCR5 expression on B cells (rMFI, mean ± SD)	44.8 ± 20.4	66.0 ± 22.6	0.002
CCR7 expression on B cells (rMFI, mean ± SD)	15.0 ± 6.4	20.9 ± 8.9	0.01
CCR7 expression on cTfh cells (rMFI, mean ± SD)	19.7 ± 6.9	23.9 ± 7.2	0.027
	CVID with polyclonal lymphoproliferation$ (n=14)	**CVID without polyclonal lymphoproliferation$ (n=19)**	**P value**
Serum IgG (z-score, mean ± SD)	3.7 ± 0.82	-2.7 ± 1.5	0.032
$IgD^-CD27^+IgA^+$ memory B cells (mean ± SD)	6.3% ± 10.8%	16.6% ± 9.7%	0.002
Naive $CD4^+$ T cells (z-score, mean ± SD)	-1.8 ± 2.2	-0.22 ± 1.9	0.036
$CD4^+$ TCM cells (z-score, mean ± SD)	1.7 ± 1.7	0.22 ± 1.7	0.032
cTfh cells (z-score, mean ± SD)	3.9 ± 3.8	0.63 ± 2.8	0.006
CXCR5 expression on B cells (rMFI, mean ± SD)	42.9 ± 19.4	56.3 ± 14.5	0.045
	PAD with autoimmunity (n=19)	**PAD without autoimmunity (n=58)**	**P value**
Serum IgA (z-score, mean ± SD)	-2.4 ± 1.1	-1.5 ± 1.7	0.012
Naive $CD4^+$ T cells (z-score, mean ± SD)	-0.93 ± 1.76	0.16 ± 1.75	0.012
$CD4^+$ RTE (z- score, mean ± SD)	-0.78 ± 1.3	0.06 ± 1.4	0.033
$CD4^+$ TCM cells (z-score, mean ± SD)	1.4 ± 1.6	-0.06 ± 1.4	0.001
cTfh cells (z-score, mean ± SD)	2.8 ± 3.9	0.21 ± 2.2	0.002
TACI expression on B cells (rMFI, mean ± SD)	166.3 ± 78.7	128.3 ± 67.1	0.052 (ns)
	CVID with autoimmunity (n=13)	**CVID without autoimmunity (n=20)**	**P value**
$CD4^+$ TCM cells (z-score, mean ± SD)	1.8 ± 1.6	0.20 ± 1.7	0.018
TACI expression on B cells (rMFI, mean ± SD)	175 ± 64	122 ± 38	0.022
	IPH with autoimmunity (n=3)	**IPH without autoimmunity (n=20)**	**P value**
$CD4^+$ TCM cells (z-score, mean ± SD)	1.2 ± 0.86	0.10 ± 1.1	0.061 (ns)
	PAD with autoimmune cytopenia	**PAD without autoimmune cytopenia (n=71)**	**P value**
TACI expression on B cells (rMFI, mean ± SD)	213.3 ± 65.4	131.3 ± 68.7	0.003
	CVID with autoimmune cytopenia	**CVID without autoimmune cytopenia (n=72)**	**P value**
TACI expression on B cells (rMFI, mean ± SD)	217 ± 72	130 ± 41	0.008

*Bronchiectasis, lung granulomata and/or lymphoid interstitial pneumonia. $Benign lymphadenopathy, hepatomegaly, and/or splenomegaly. cTfh: circulating follicular helper T, CVID: common variable immunodeficiency, Ig: immunoglobulin, IPH: idiopathic primary hypogammaglobulinemia, ns: not significant, PAD: primary antibody deficiency, rMFI: relative mean fluorescence intensity, RTE: recent thymic emigrants, SD: standard deviation, TCM: central memory T.

SUPPLEMENTARY FIGURES

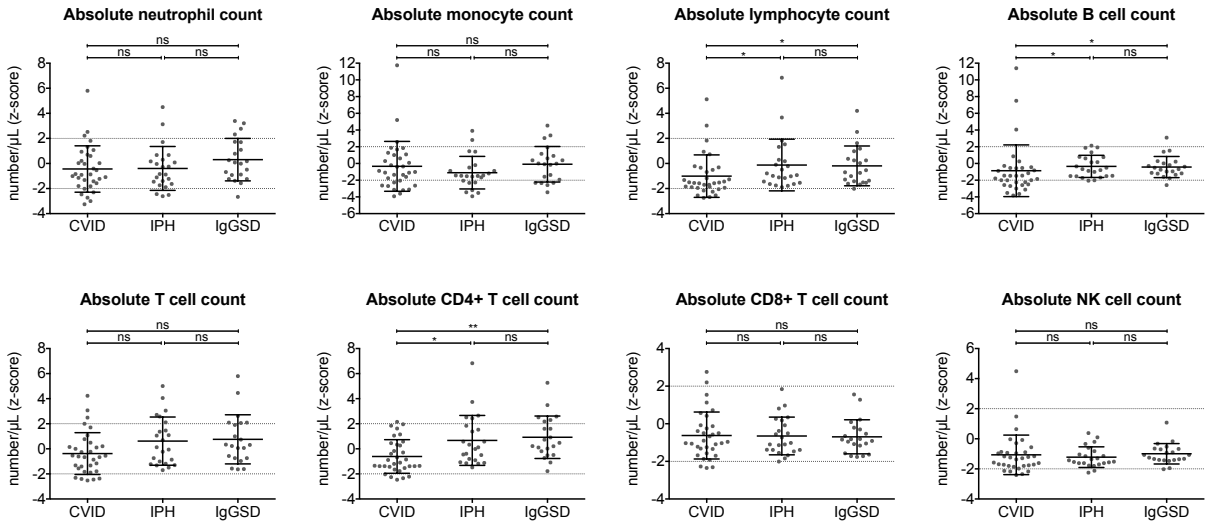


Figure S1. Absolute white blood cell counts in patients. Absolute white blood cell counts in CVID, IPH and IgGSD patients determined in routine lab evalutions. Graphs represent mean ± SD. Cell counts were expressed as z-scores to adjust for age. Values normal for age have a z-score between -2 and 2 (dotted lines). CVID: common variable immunodeficiency, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia. * p≤0.05, ** p≤0.01, *** p≤0.001, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

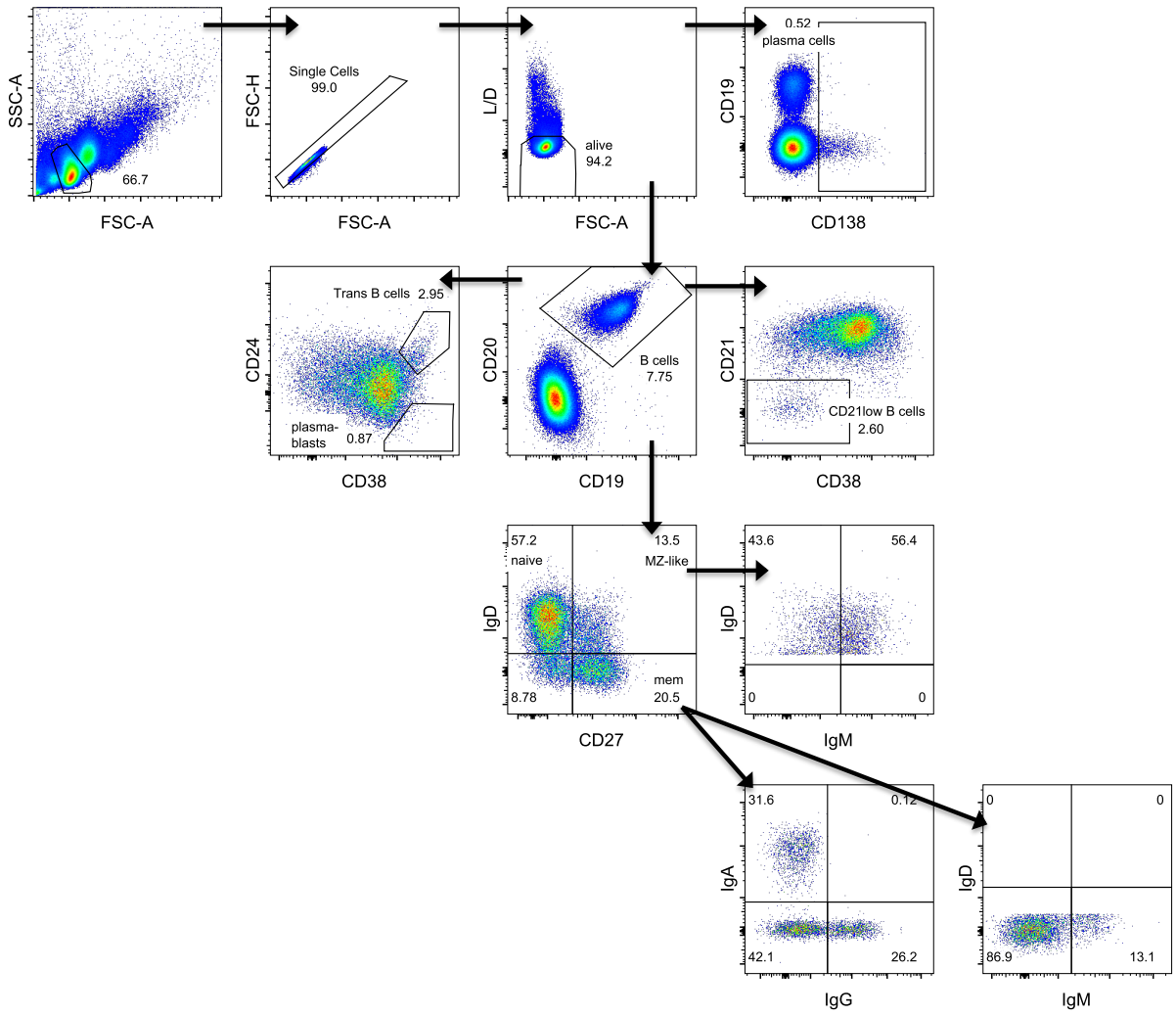


Figure S2. Representative flow cytometric analysis of B cell subsets in an adult healthy control. PBMCs were sequentially gated on lymphocytes (gating out debris and monocytes), single cells (gating out doublets) and alive cells (gating out dead cells). B cells were gated as $CD19^{+}CD20^{+}$ in alive cells. Plasma cells were gated as $CD138^{+}CD19^{low}$ in alive cells. Within B cells, transitional (trans) B cells were gated as $CD24^{high}CD38^{high}$, plasmablasts as $CD38^{high}CD24^{-}$, $CD21^{low}$ B cells as $CD21^{low}CD38^{low}$, memory (mem) B cells as $IgD^{-}CD27^{+}$, naive B cells as $IgD^{+}CD27^{-}$, and marginal zone (MZ)-like B cells as $IgD^{+}CD27^{+}$. Within $IgD^{+}CD27^{+}$ marginal zone-like B cells, IgM^{+} marginal zone-like B cells were gated based on a Fluorescence Minus One (FMO). Within $IgD^{-}CD27^{+}$ memory B cells, IgG^{+}, IgA^{+} and IgM^{+} memory B cells were gated based on an FMO. L/D: live/dead marker.

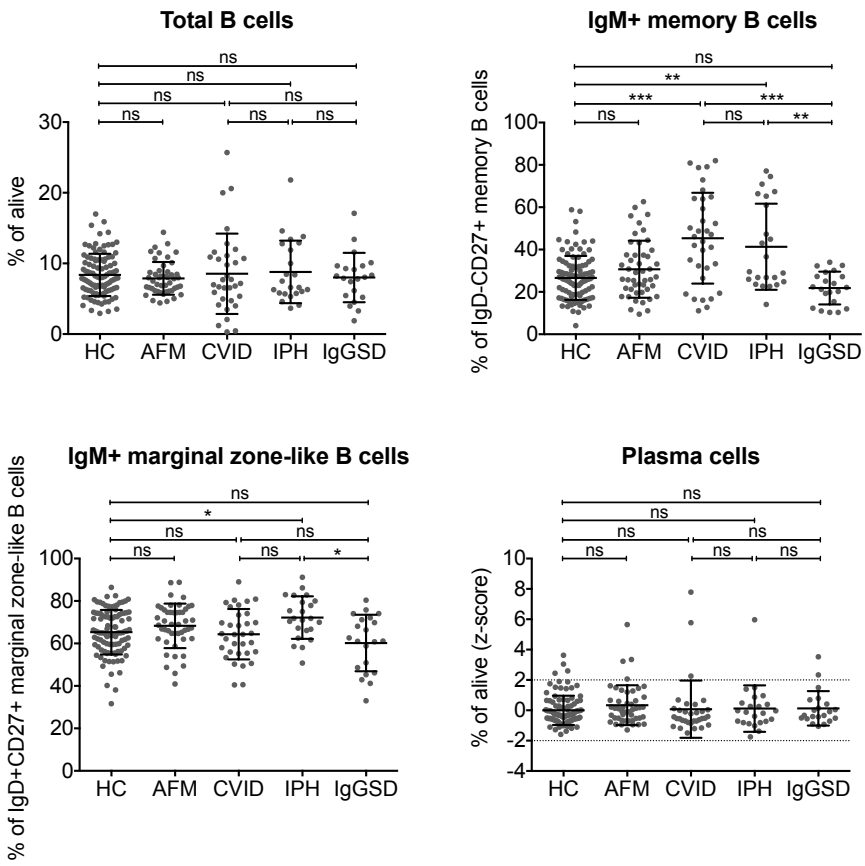


Figure S3. Total B cells, IgD$^-$CD27$^+$IgM$^+$ memory B cells, IgM$^+$IgD$^+$CD27$^+$ marginal zone-like B cells, and plasma cells. B cells were gated as CD19$^+$CD20$^+$ and plasma cells as CD138$^+$CD19^{low} in alive cells. IgD$^-$CD27$^+$ memory B cells and IgD$^+$CD27$^+$ marginal zone-like B cells were gated within B cells. Graphs represent mean ± SD. Plasma cells were expressed as z-scores to adjust for age. Values normal for age have a z-score between -2 and 2 (dotted lines). AFM: asymptomatic family member, CVID: common variable immunodeficiency, HC: healthy control, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia. * p≤0.05, ** p≤0.01, *** p≤0.001, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

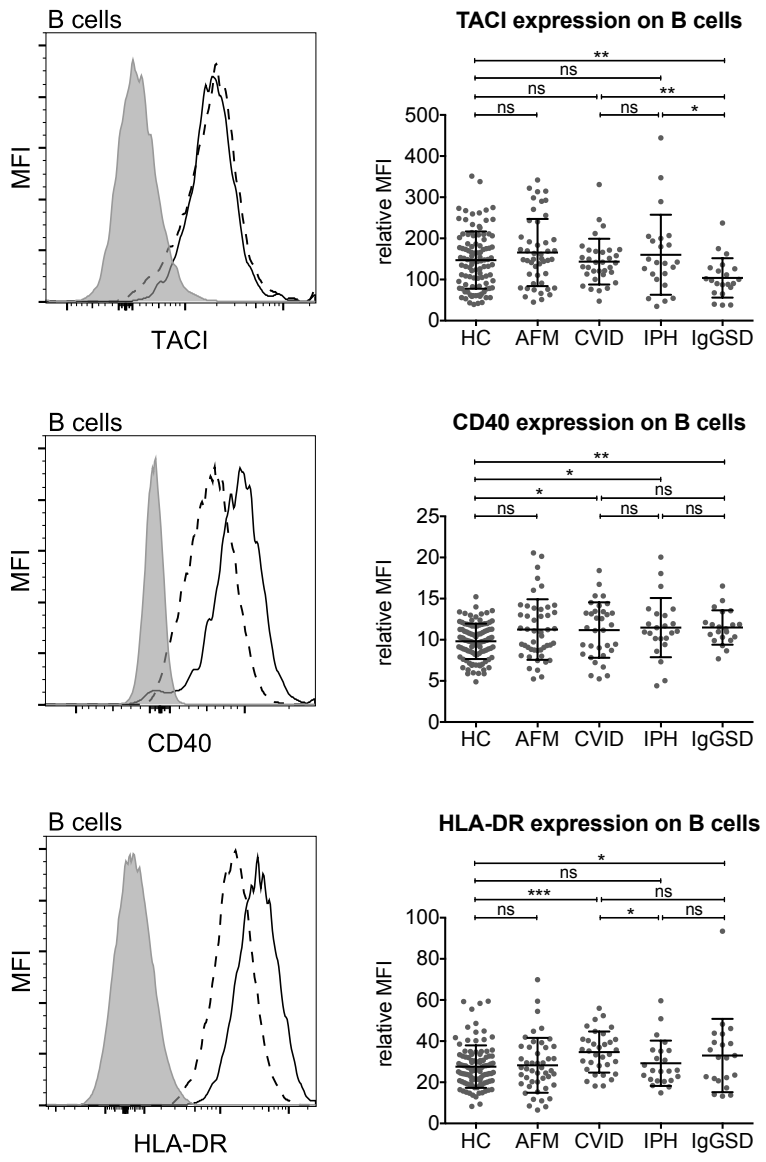


Figure S4. TACI, CD40 and HLA-DR expression on B cells. Graphs represent mean ± SD. Representative flow cytometric analysis is shown on the left. Full black line represents CVID patient, dashed black line represents HC. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population (black line) by the MFI of the Fluorescence Minus One (FMO) population (gray). AFM: asymptomatic family member, CVID: common variable immunodeficiency, HC: healthy control, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia. * p≤0.05, ** p≤0.01, *** p≤0.001, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

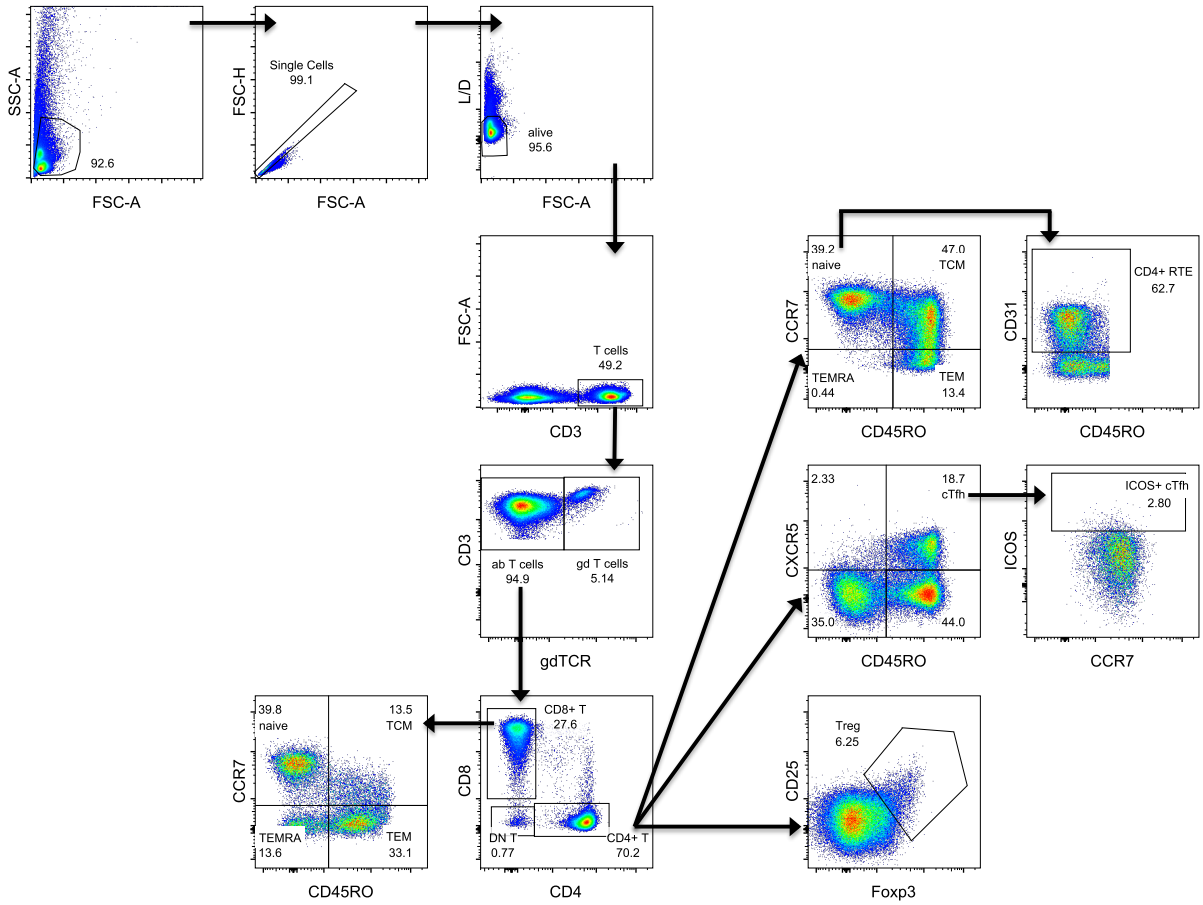


Figure S5. Representative flow cytometric analysis of naive and memory T cell subsets, DN T cells and Treg cells in an adult healthy control. PBMCs were sequentially gated on cells (gating out debris), single cells (gating out doublets) and alive cells (gating out dead cells). Total T cells were gated as $CD3^+$ in alive cells. αβ T cells were gated as $\gamma\delta TCR^-$ and γδ T cells as $\gamma\delta TCR^+$ in total T cells. $CD4^+$ and $CD8^+$ T cells were gated in αβ T cells. Double negative (DN) T cells were gated as $CD4^-$ $CD8^-$ in αβ T cells. In $CD4^+$ and $CD8^+$ T cells, naive cells were gated as $CD45RO^-CCR7^+$, central memory cells (TCM) as $CD45RO^+CCR7^+$, effector memory cells (TEM) as $CD45RO^+CCR7^-$, and terminally differentiated cells (TEMRA) as $CD45RO^-CCR7^-$. In naive $CD4^+$ T cells, recent thymic emigrants (RTE) were gated as $CD31^+$. Regulatory T (Treg) cells were gated as $CD25^+Foxp3^+$ in $CD4^+$ T cells based on Fluorescence Minus One (FMO). Circulating follicular helper T (cTfh) cells were gated as $CXCR5^+CD45RO^+$ in $CD4^+$ T cells. Within cTfh cells, $ICOS^+$ cTfh cells were gated based on FMO. L/D: live/dead marker.

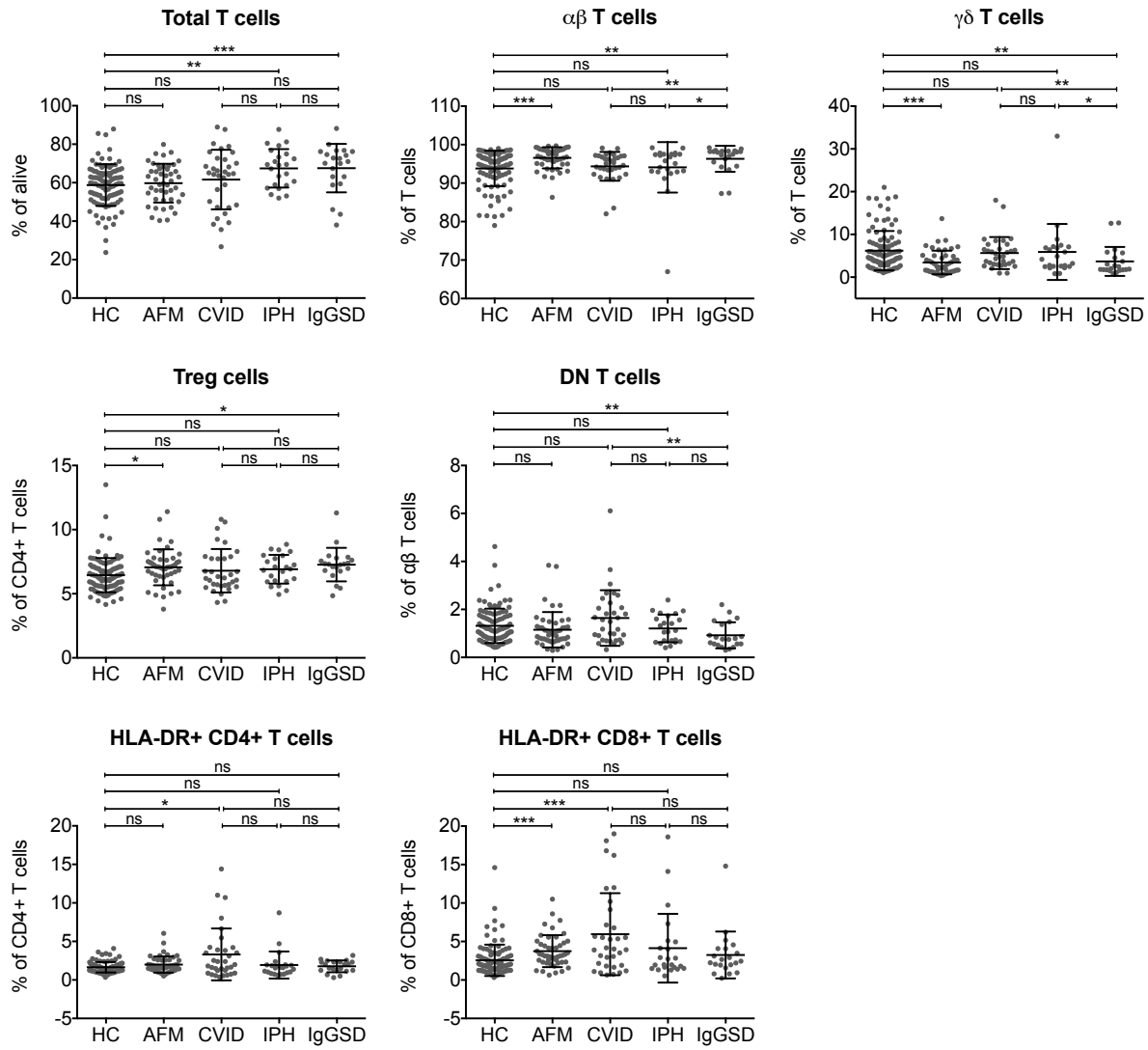


Figure S6. T cell subsets: total T cells, αβ T cells, γδ T cells, Treg cells, DN T cells, HLA-DR$^+$CD4$^+$ and HLA-DR$^+$CD8$^+$ T cells. Total T cells were gated as CD3$^+$ in alive cells. αβ T cells were gated as γδTCR$^-$ and γδ T cells as γδTCR$^+$ in total CD3$^+$ T cells. Regulatory T (Treg) cells were gated as CD25$^+$Foxp3$^+$ in CD4$^+$ T cells. Double negative (DN) T cells were gated as CD4$^-$CD8$^-$ in αβ T cells. HLA-DR$^+$ cells were gated in CD4$^+$ and CD8$^+$ T cells. Graphs represent mean ± SD. AFM: asymptomatic family member, CVID: common variable immunodeficiency, HC: healthy control, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia. * p≤0.05, ** p≤0.01, *** p≤0.001, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

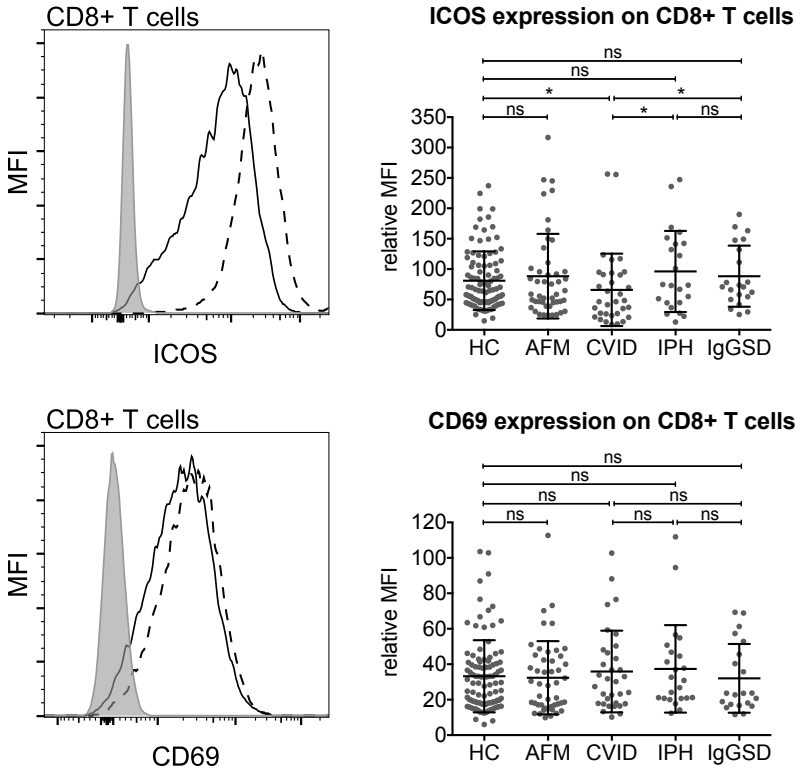


Figure S7. ICOS upregulation on stimulated CD8$^+$ T cells. ICOS and CD69 expression on CD8$^+$ T cells stimulated with PHA for 72 hours. CD69 was used as a positive control for T cell activation. Graphs represent mean ± SD. Representative flow cytometric analysis is shown on the left. Full black line represents CVID patient, dashed black line represents HC. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population (black line) by the MFI of the Fluorescence Minus One (FMO) population (gray). AFM: asymptomatic family member, CVID: common variable immunodeficiency, HC: healthy control, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia. * p≤0.05, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

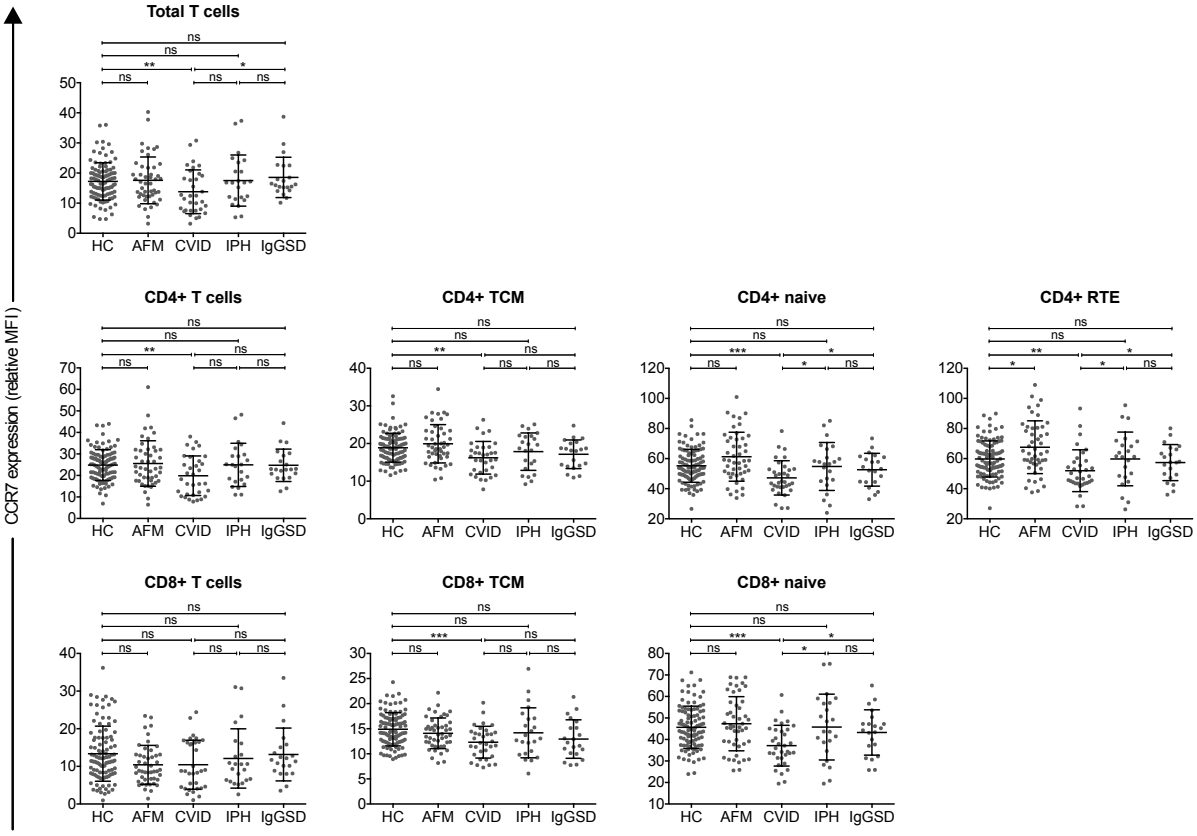


Figure S8. CCR7 expression on T cell subsets. Total T cells were gated as $CD3^+$ in alive cells. $CD4^+$ and $CD8^+$ T cells were gated in $\alpha\beta$ T cells. Within $CD4^+$ and $CD8^+$ T cells, central memory cells (TCM) were gated as $CD45RO^+CCR7^+$ and naive cells as $CD45RO^-CCR7^+$. In naive $CD4^+$ T cells, recent thymic emigrants (RTE) were gated as $CD31^+$. Relative mean fluorescence intensity (MFI) of CCR7 was calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Graph represents mean ± SD. AFM: asymptomatic family member, CVID: common variable immunodeficiency, HC: healthy control, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia. * p≤0.05, ** p≤0.01, *** p≤0.001, ns not significant (Mann-Whitney test with Bonferonni's correction for multiple comparisons).

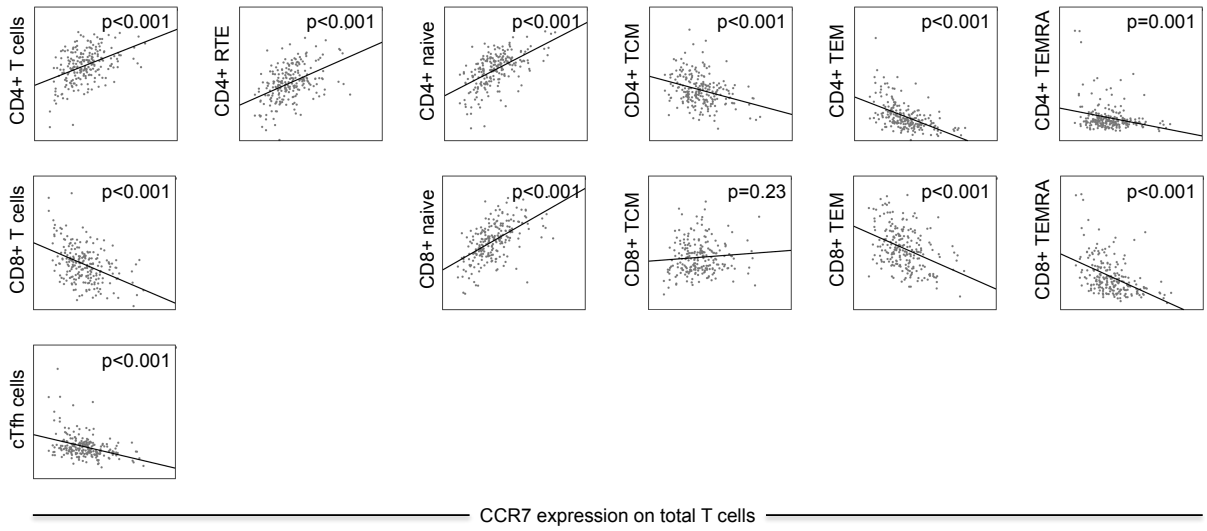


Figure S9. Correlation between CCR7 expression on total T cells and levels of T cell subsets. Total T cells were gated as $CD3^+$ in alive cells. $CD4^+$ and $CD8^+$ T cells were gated in $\alpha\beta$ T cells. Within $CD4^+$ and $CD8^+$ T cells, naive cells were gated as $CD45RO^-CCR7^+$, central memory cells (TCM) as $CD45RO^+CCR7^+$, effector memory cells (TEM) as $CD45RO^+CCR7^-$, and terminally differentiated cells (TEMRA) as $CD45RO^-CCR7^-$. In naive $CD4^+$ T cells, recent thymic emigrants (RTE) were gated as $CD31^+$. Circulating follicular helper T (cTfh) cells were gated as $CXCR5^+CD45RO^+$ in $CD4^+$ T cells. All naive and memory T cell subsets, including cTfh cells, were expressed as z-scores to adjust for age. CCR7 expression on total T cells is the relative mean fluorescence intensity (MFI) of CCR7 on total T cells, calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Correlations were calculated with Spearman Rank Correlation.

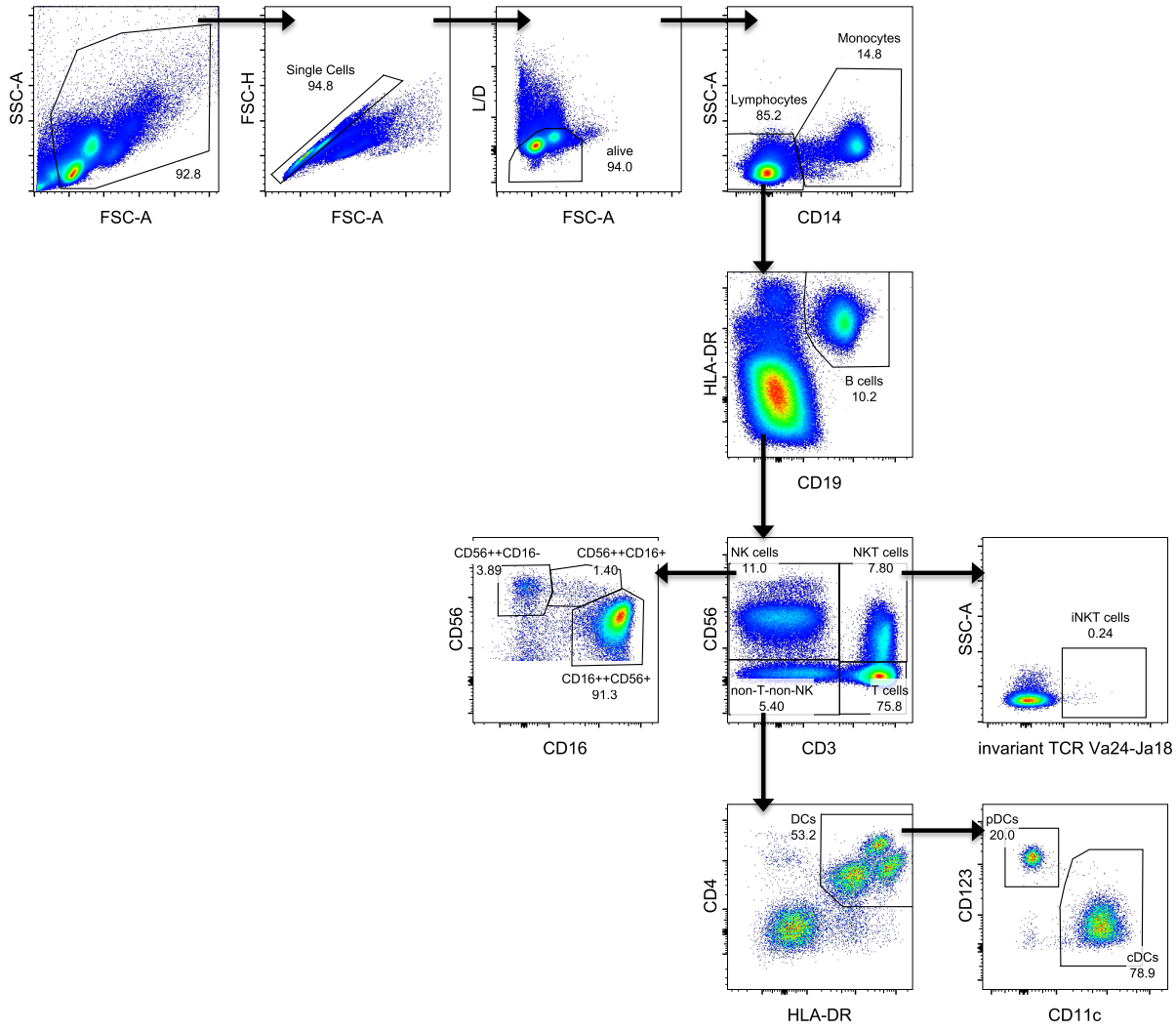


Figure S10. Gating strategy for innate immune cell subsets in an adult healthy control. PBMCs were sequentially gated on cells (gating out debris), single cells (gating out doublets) and alive cells (gating out dead cells). Lymphocytes and monocytes were gated on SSC and CD14. B cells were gated as $CD19^{+}HLA\text{-}DR^{+}$ in lymphocytes; thereafter gating was continued on the NOT-gate. T cells were gated as $CD56^{-}CD3^{+}$, natural killer (NK) cells as $CD3^{-}CD56^{+}$ and natural killer T (NKT) cells as $CD3^{+}CD56^{+}$ in $CD19^{-}$ lymphocytes. NK cell subsets were determined by their relative expression of CD56 and CD16. Invariant NKT (iNKT) cells were gated as invariant TCR (TCR Vα24-Jα18) positive in NKT cells. Dendritic cells (DCs) were gated as $CD4^{+}HLA\text{-}DR^{+}$ in non-T-non-NK cells. Within DCs, conventional DCs (cDCs) were gated as $CD11c^{+}CD123^{-}$ and plasmacytoid DCs (pDCs) as $CD123^{+}CD11c^{-}$. L/D: live/dead marker.

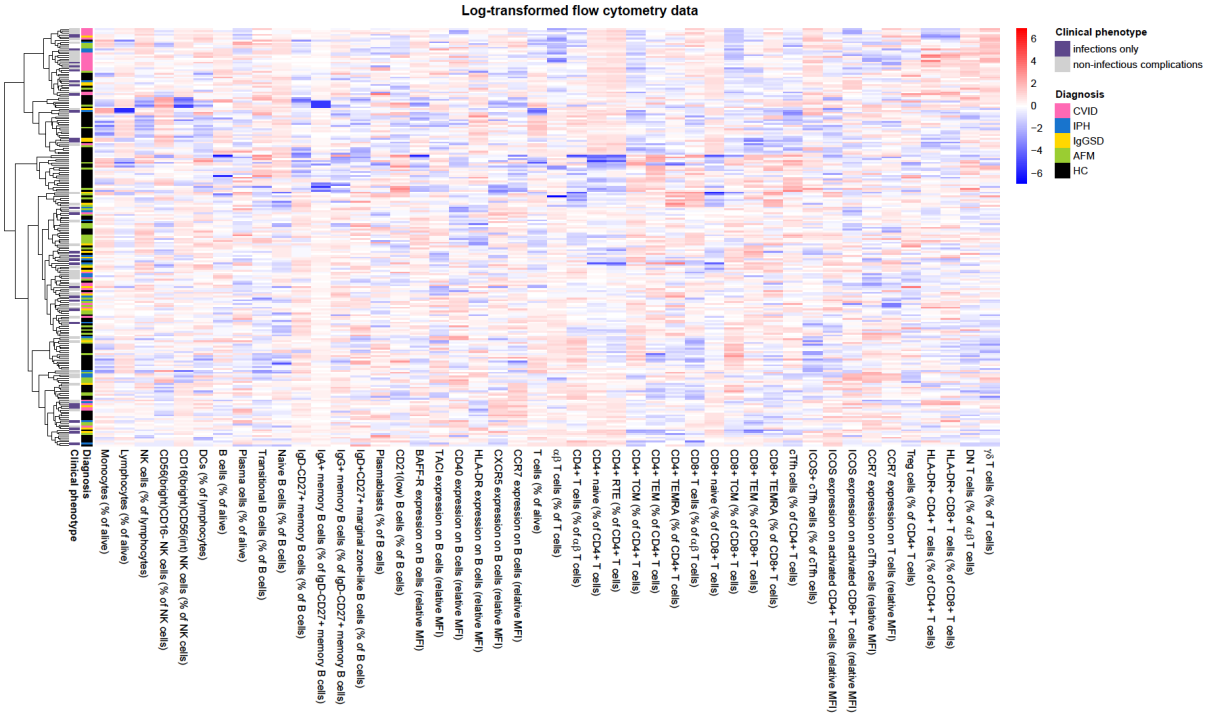


Figure S11. Unsupervised computational clustering analysis: hierarchical clustering and heatmap. Unsupervised clustering of the study cohort based on 46 flow cytometric parameters. Study subjects are stratified according to diagnosis and patients are also stratified according to clinical phenotype (infections only versus non-infectious complications). Hierarchical clustering, plotted in the dendrogram on the left hand side, shows that subjects do not cluster according to diagnosis or clinical phenotype. The heatmap of log-transformed flow cytometric data does not reveal subgroups among patients nor distinguish patient from AFM or HC. AFM: asymptomatic family member, cTfh: circulating follicular helper T, CVID: common variable immunodeficiency, DCs: dendritic cells, DN T: double negative T, HC: healthy control, IgGSD: IgG subclass deficiency, IPH: idiopathic primary hypogammaglobulinemia, MFI: mean fluorescence intensity, NK: natural killer, RTE: recent thymic emigrants, TCM: central memory T, TEM: effector memory T, TEMRA: effector memory RA T, Treg: regulatory T.

SUPPLEMENTARY REFERENCES

1. R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.
2. Kolde R (2015). pheatmap: Pretty Heatmaps. R package version 1.0.7. http://CRAN.R-project.org/package=pheatmap
3. Clark-Carter D (2005). Encyclopedia of Statistics in Behavioral Science. Hoboken: John Wiley and Sons, Ltd.
4. Wehr C, Kivioja T, Schmitt C, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood. 2008;111(1):77-85.
5. Giovannetti A, Pierdominici M, Mazzetta F, et al. Unravelling the complexity of T cell abnormalities in common variable immunodeficiency. J Immunol. 2007;178(6):3932-3943.

Chapter 4

Genetic testing in patients with primary antibody deficiencies: overview of the Ghent cohort

4 GENETIC TESTING IN PATIENTS WITH PRIMARY ANTIBODY DEFICIENCIES: OVERVIEW OF THE GHENT COHORT

4.1 Introduction

A human has about 20,000 to 25,000 protein-coding genes, constituting 1-2% of the entire genome (i.e. the complete set of genetic material).[1] The non-protein-coding part of the genome (~98%) also has important biological functions, such as regulation of gene expression. However, the exact function of non-coding DNA remains largely elusive. In medicine, genetic testing involves the analysis of genetic material to obtain information related to an individual's disease status. Cytogenetic techniques are used to screen for chromosomal alterations, and molecular genetic techniques to analyze DNA sequence alterations in genes (i.e. mutation analysis).[1]

Genetic testing in primary immunodeficiencies (PIDs) has, so far, mainly focused on DNA sequence alterations in protein-coding genes.[2] Since the 1980s, Sanger sequencing has been the gold standard for DNA-based screening of disease genes.[3] The past decade, massively parallel or next-generation sequencing (NGS) technologies have been developed, enabling the simultaneous analysis of millions of DNA sequences.[4] Like for other genetically determined disorders, NGS technologies have facilitated the discovery of numerous novel PID-associated genes.[2] Still, little progress has been made in patients with common variable immunodeficiency (CVID) and related primary antibody deficiencies (PADs) (see also chapter 1, section 1.6).[5,6]

The **goal of this chapter** is to outline the types of genetic testing that have, so far, been performed in the Ghent PAD cohort of 70 index patients and the results obtained by each approach. In addition, we propose a strategy for genetic testing in patients with CVID and related antibody disorders.

4.2 Methods

4.2.1 Recruitment of the study cohort

Seventy index patients with PADs were recruited between October 2013 and November 2015 at the pediatrics, adult hematology and adult pulmonology departments at Ghent University Hospital.[7] The definitions of the clinical diagnoses were determined before start of recruitment and, if possible, based on the diagnostic criteria formulated by the European Society for Immunodeficiencies (ESID) and the Pan-American Group for Immunodeficiency

(PAGID) in 1999 (Table 1) (see also Chapter 1, section 1.3).[8] In addition, affected and unaffected family members of the index patients were also included in the study.

The study was approved by the ethical committee of Ghent University Hospital (2012/593). All subjects (or their parents in case of pediatric subjects) provided written informed consent to participation in the study, in accordance with the Helsinki Declaration of 1975.

Table 1. Diagnostic criteria for the Ghent cohort of PAD patients.

Diagnosis	Criteria	Ref.
Common variable immunodeficiency (CVID)	Serum IgG at least 2 SD below mean for age AND serum IgA and/or IgM at least 2 SD below mean for age AND poor response to protein and/or polysaccharide vaccines AND other causes of antibody deficiency have been excluded*	8
Idiopathic primary hypogammaglobulinemia (IPH)	Serum IgG at least 2 SD below mean for age AND not fulfilling CVID criteria AND other causes of antibody deficiency have been excluded*	9
IgG subclass deficiency (IgGSD)	Serum IgG2 and/or IgG3 at least 2 SD below mean for age AND normal total serum IgG and serum IgM AND normal or decreased serum IgA AND good or poor response to protein and/or polysaccharide vaccines AND other causes of antibody deficiency have been excluded*	10
Selective IgM deficiency (sIgMD)	Serum IgM at least 2 SD below mean for age AND normal serum IgG and IgA AND good response to protein and polysaccharide vaccines AND other causes of antibody deficiency have been excluded*	11
Selective IgA deficiency (sIgAD)	Serum IgA at least 2 SD below mean for age AND normal serum IgG and IgM AND good response to protein and polysaccharide vaccines AND other causes of antibody deficiency have been excluded*	8,12
Agammaglobulinemia	Less than 2% circulating B cells AND onset of recurrent bacterial infections before 5 years of age AND serum IgG, IgA and IgM at least 2 SD below mean for age AND poor response to protein and/or polysaccharide vaccines AND other causes of antibody deficiency have been excluded*	8,13
Syndromic PAD	Serum IgG, IgA and/or IgM at least 2 SD below mean for age AND congenital malformations, dysmorphic features and/or other phenotypical abnormalities not directly associated with the immune defect AND secondary causes of antibody deficiency have been excluded*	14

All laboratory parameters were measured at least twice. *Defined by ESID/PAGID 1999.[8] Ig: immunoglobulin, PAD: primary antibody deficiency, Ref.: References, SD: standard deviations.

4.2.2 DNA extraction

Blood samples were retrieved from all subjects included in the study. Genomic DNA (gDNA) was isolated from whole blood leukocytes using the QIAamp DNA Mini Kit (QIAGEN), Gentra Puregene Blood Kit (QIAGEN), or Wizard Genomic DNA Purification Kit (Promega), according to manufacturer's instructions.

The QIAamp procedure yields lower amounts of gDNA (~nanograms) typically used to sequence a small number of targets after polymerase chain reaction (PCR)-based

enrichment. The Gentra Puregene and Promega methods provide higher amounts of gDNA (~micrograms) as needed for whole exome sequencing (WES).

4.2.3 Polymerase chain reaction (PCR)-based target enrichment

To analyze the DNA sequence of a gene or target of interest, this sequence needs to be amplified (i.e. enriched) before genetic testing can be performed. PCR is a commonly used technique to generate millions of copies of a particular DNA sequence.[1] To selectively enrich the DNA segment of interest, the PCR technique makes use of forward and reverse primers. Primers are custom-made oligonucleotide sequences (~20 base pairs [bp]) designed in such a manner that they specifically flank each side of the target DNA region.[1] In this study, primers for amplification and sequencing were manually designed by use of Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)[15], based on reference DNA sequences obtained from UCSC Genome Browser (https://genome.ucsc.edu).

Singleplex PCR reactions were performed according to an in-house optimized ("generic") protocol.[16] In summary, each PCR reaction mixture consisted of 2.5 µL gDNA template (20 ng/µL), 2.5 µL amplification primers (forward and reverse primer mixed, 2 µM), and 5 µL KAPA2G Robust Hotstart Ready Mix (KAPA Biosystems). Next, PCR reactions were run on a MJ Research PTC-200 Thermal Cycler (MJ Research Inc.): an initial denaturation step at 95°C for 3 min; 35 cycles of (1) denaturation at 95°C for 15 sec, (2) annealing at 60°C for 10 sec, and (3) extension at 72°C for 15 sec; and a final extension step at 72°C for 1 min. Afterwards, amplification specificity and quality of the PCR product were assessed using automated capillary electrophoresis (LabChip GX, PerkinElmer).

PCR-based target enrichment is most suitable for sequencing of a small number of genes, and was therefore used in combination with Sanger sequencing or NGS-based targeted resequencing (see below).

4.2.4 Sanger sequencing

DNA sequencing entails the determination of the exact nucleotide composition of the DNA segment of interest. The sequencing method developed by Frederic Sanger (Sanger sequencing) is based on chain termination by the random incorporation of fluorescently-labeled dideoxynucleotides during a classical PCR reaction.[3] After several cycles of denaturation, annealing and elongation, the final sequencing product is a mixture of DNA fragments terminated at different lengths. These fragments are separated according to their length by means of capillary electrophoresis, after which a laser detects the fluorescent labels of the incorporated dideoxynucleotides at the end of each fragment. The generated

output is a sequence read (electropherogram) showing the exact nucleotide composition of the target of interest. By comparing this output to the human reference sequence, nucleotide variations (e.g. pathogenic mutations, benign variants) can be identified.[1]

In this study, PCR products of the prior enrichment step were enzymatically purified with Exonuclease I and Antarctic phosphatase (both New England BioLabs Inc.). Purified PCR products were then Sanger sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on a 3730xl DNA Analyzer (Applied Biosystems). Sequence reads were analyzed with SeqScape v2.5 (ThermoFisher Scientific).

4.2.5 Next-generation sequencing (NGS)

4.2.5.1 Sequencing technology

Several NGS technologies have been developed in recent years. In this thesis, we used the Illumina sequencing technology known as sequencing by synthesis, which is the most widely adopted NGS technique.[17] gDNA first undergoes several preparation steps before it can be sequenced (i.e. library preparation). In particular, DNA is randomly fragmented and known adaptor sequences are ligated at both ends of each DNA fragment. The adaptor-ligated DNA fragments are then amplified with PCR using primers that carry a specific index. With this index, the DNA fragments can later be traced back to the original sample when fragments of different samples are sequenced simultaneously. Next, the DNA library is denatured and applied to a glass flow cell of the sequencing instrument. The adaptors at the end of the DNA fragments hybridize with complementary oligonucleotides on the flow cell, followed by multiple cycles of so-called bridge amplification.[17] Single nucleotides are detected continually as they are incorporated into the growing DNA strands. To enable this, Illumina has designed fluorescently labeled reversible terminator nucleotides. During each sequencing cycle, the fluorescent signals of the newly incorporated nucleotides are detected. Afterwards, the fluorophore and blocking site of the nucleotides are cleaved and washed away, allowing the incorporation of new nucleotides in the next cycle. The final output consists of a multitude of sequencing reads, which are aligned or mapped to the human reference genome and subsequently analyzed using specific software tools. “Read depth” refers to the number of sequence reads that include a particular nucleotide. “Coverage” is sometimes used as a synonym for read depth, but may also refer to the percentage of target regions that is sequenced to a preset read depth (e.g. 98% of target regions have a minimal read depth of 20x).[2]

4.2.5.2 NGS-based targeted resequencing

NGS technologies can be used to sequence a selected panel of genes or targets, a method known as targeted resequencing. For this, the selected genes of interest need to be isolated and enriched. In-house, a platform for targeted resequencing was developed using singleplex PCR for target enrichment.[16] In brief, targets of different samples (individuals) were first enriched with singleplex PCR as explained in section 4.2.3. Next, several pooling steps, DNA fragmentation and library preparation were performed (Nextera XT DNA Library Preparation Kit, Illumina). Finally, sequencing was done on a MiSeq instrument (Illumina).

4.2.5.3 Whole exome sequencing (WES)

Protein-coding genes consist of exons and introns. Exons contain the actual code for protein synthesis whereas introns are spliced out during RNA maturation. Whole exome sequencing (WES) aims to sequence all coding exons (collectively called exome) in a single person. The exonic regions are enriched via a "capture" procedure.[18] In particular, adaptor-ligated DNA (see section 4.2.5.1) is hybridized with oligonucleotide probes that bind or capture the exonic regions of the DNA fragments. These probes are then pulled-down by specific beads, taking the captured exonic sequences with them. Next, the captured regions are, again, amplified with PCR, followed by massively parallel sequencing. The main limitation of the capture-based enrichment method is that not all exons are sufficiently covered, especially those with a high GC content. Furthermore, off-target capturing is often seen for highly homologous regions.[16]

In this thesis, whole exome enrichment or capturing was performed with SureSelectXT Human All Exon (+/-UTR) kits (Agilent Technologies). Massively parallel sequencing (paired-end 150 cycles) was performed on a NextSeq 500 (Illumina).

4.2.5.4 Analysis of NGS data

Read mapping against the human genome reference sequence (NCBI, GRCh37), and post-mapping duplicate read removal, quality-based variant calling and coverage analysis were performed with CLC Genomics Workbench v6.0.4 (CLC bio). Called variants with read depth ≥ 3 were annotated with Alamut Batch (Interactive Biosoftware). Only variants with population frequencies less than 10% were considered, according to public databases NCBI dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), NHLBI Exome Sequencing Project - Exome Variant Server (http://evs.gs.washington.edu/EVS/), ExAC Browser (http://exac.broadinstitute.org/), and 1000 Genomes Project Browser (http://browser.1000genomes.org/). Variants were further prioritized based on allele frequency, *in silico* pathogenicity prediction scores (e.g. SIFT, PolyPhen2, MAPP,

MutationTaster, Grantham distance, Align GVGD), nucleotide and amino acid conservation scores (e.g. phyloP, PhastCons), and biological relevance.[19] Both Mendelian and non-Mendelian inheritance patterns were taken into account. Afterwards, variants of interest were further evaluated using Alamut Visual mutation interpretation software v2.7 rev. 1 (Interactive Biosoftware), Ingenuity Variant Analysis (QIAGEN, 2015 Release Spring), CADD scores v1.3 (http://cadd.gs.washington.edu/home), genome Aggregation Database (gnomAD) Browser (http://gnomad.broadinstitute.org), literature search, segregation analysis in available family members, and frequency in an in-house database containing variants of more than 1000 exomes at time of analysis.
Validation of variants and segregation analysis were performed by means of Sanger sequencing (see section 4.2.4).

4.2.6 Microarray-based comparative genomic hybridization (array CGH)

Microarray-based comparative genomic hybridization (array CGH) is a technique to detect copy number variations (CNVs), i.e. deletions or duplications of DNA regions > 1 kb, on a genome-wide level.[1] Patient and control gDNA are fluorescently labeled with a different color. After mixing patient and control samples, DNA is hybridized to known oligonucleotides fixed on an array. For each location on the array, fluorescent signals of patient and control are compared, allowing the identification of deleted or duplicated regions in the patient.[1]
In this thesis, we used an 180K oligonucleotide array with an average genome-wide resolution of 100 kb (SurePrint G3 Human CGH Microarray Kit, Agilent Technologies). Hybridizations were performed according to manufacturer's instructions. Results were analyzed using arrayCGHbase.[20]

4.2.7 Homozygosity mapping

Homozygosity mapping or identity-by-descent (IBD) mapping is used to determine large homozygous genomic regions in patients with a consanguineous background, based on the assumption that the causal mutation will be homozygous and located in a run of homozygosity identical by descent.[21] Homozygous regions can be distinguished by use of a genome-wide single nucleotide polymorphism (SNP) chip array. Patient gDNA is fragmented and hybridized on a chip array coated with oligonucleotides. These oligonucleotides correspond to thousands of SNPs equally spread out over the entire genome. Next, the genotype of each SNP (hetero- or homozygous) is determined, enabling the localization of larger homozygous regions within the patient genome. To narrow down the number and length of the homozygous regions of interest, homozygosity mapping can also be performed

in affected and/or non-affected siblings of the patient. Importantly, SNP chip arrays can also be used for CNV analysis.[1]

In this thesis, the HumanCytoSNP-12 BeadChip platform (Illumina) was used for homozygosity mapping in patients from consanguineous families. Homozygous regions larger than 1 Mb were retained for further analysis.

4.3 Results

4.3.1 The Ghent PAD cohort: characteristics and genetic approach

The **Ghent PAD cohort** consisted of **70 index patients** including 33 males and 37 females. About 90% of the index patients were clinically diagnosed with CVID (n=28), idiopathic primary hypogammaglobulinemia (IPH) (n=19), or IgG subclass deficiency (IgGSD) (n=16). The remaining 10% included selective IgM deficiency (sIgMD) (n=1), selective IgA deficiency (sIgAD) (n=1), agammaglobulinemia (n=1) and syndromic PAD (n=4). All patients were Caucasian and from European or North African descent. The average age at time of inclusion was 31.8 years (range 22 months to 86 years). One patient was born to healthy consanguineous parents. Twenty-one patients (30%) had a family history suggestive of PID; symptomatic family members were available in 16 out of these 21 cases.

In total, **98 family members of the index patients** were also included in the study: 82 (84%) first-degree (parents, siblings, children) and 16 (16%) second- and third-degree (grandparents, aunts, uncles, first cousins) relatives. Twenty-five out of 98 (25.5%) included family members were given a clinical diagnosis of PAD, which were similar to the diagnoses of the corresponding index patients.

As a first approach, candidate genes based on the patients' phenotype were screened by means of Sanger sequencing. Furthermore, array CGH was used to screen for large (> 100 kb) CNVs. Once NGS technologies became available in-house, the genetic testing toolbox was expanded with targeted resequencing of selected genes and WES. In the following paragraphs, we aimed to outline the **types of genetic testing** that were, so far, performed in our cohort and the **results obtained by each approach**. It is important to note that some types of genetic testing were only performed in a subset of patients, and that genetic analyses are still ongoing in this cohort. Detailed discussion of the mentioned genes and disease entities was outside the scope of this chapter.

4.3.2 Array CGH

Array CGH (resolution ~100 kb) is a valuable technique to screen for large CNVs, which would be missed using PCR-based approaches (Sanger sequencing, targeted NGS) or WES. In our cohort, array CGH was performed in 31 index patients, including all four patients with syndromic PAD.

Patient 1 had a heterozygous deletion involving the distal section of the long arm of chromosome 18 (Table 2), rendering him a genetic diagnosis of 18q deletion syndrome.[22] The patient presented with antibody deficiency, recurrent infections, intellectual disability and other syndromic features (Table 3). Variable degrees of antibody deficiency were known to be associated with 18q deletion syndrome, and both the immunological and extra-immunological phenotype of the patient matched the underlying genotype (Table 3).[22-24]
Array CGH findings in other patients were considered not to be pathogenic by experienced clinical geneticists (data not shown). Hence, the array CGH approach had a success rate of 3% (1/31) (Figure 1, left graph).

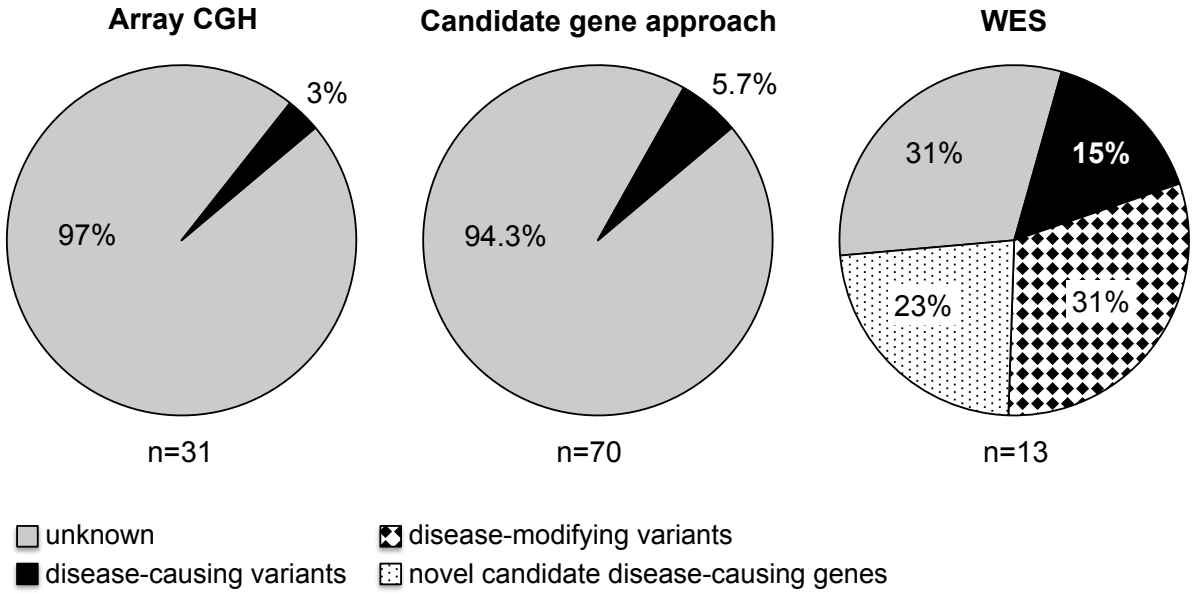


Figure 1. Comparison of three methods of genetic testing in the Ghent PAD cohort. Microarray-based comparative genomic hybridization (array CGH) was performed in 31 index patients, screening of candidate genes in all 70 index patients (2 to 5 genes per patient), and whole exome sequencing (WES) in 13 index patients. The percentage of tested patients for which the specified result was obtained, is indicated for each method.

Table 2. Disease-causing and -modifying variants identified in the Ghent PAD cohort.

Patient	Gene (OMIM identifier) or chromosome	Inheritance	Transcript	Coding change	Protein change	ClinVar (clinical significance)	PMID	Computational pathogenicity predictions of novel variants
1	Chromosome 18q	-	Molecular karyotype: 46,XY,del(18)(q21.1q23)			-	17632778	-
2	*BTK* (*300300)	XR	NM_001287344	c.1661G>A	p.Arg520Gln	RCV000012131.11 (pathogenic)	7849721, 7880320	-
3	*GATA2* (*137295)	*De novo*	NM_032638	c.1017+572C>T (= i5C>T)	-	-	23502222, 25359990	-
4	*NFKB1* (*164011)	AD	NM_003998	c.191G>T	p.Gly64Val	- *(novel variant)*	-	Highly conserved nucleotide and amino acid, MutationTaster: disease causing, CADD phred score: 29 (MSC: 3.313)
5	*RNU4ATAC* (*601428)	AR	NR_023343	c.13C>T	-	-	26522830	-
				c.116A>T	-	-	-	See Chapter 5 [$]
6	*KMT2A* (+159555)	AD	NM_001197104	c.10835+1G>A	-	- *(novel variant)*	-	See Chapter 5 [$]
7	*IKZF1* (*603023)	AD	NM_006060	c.136delA	p.S46Afs*14	- *(novel variant)*	-	See Chapter 6 [$]
8 + 9	*TNFRSF13B* (TACI) (*604907)	AD	NM_012452	c.310T>C	p.Cys104Arg	Variation ID 5302 (benign / pathogenic)	16007086, 16007087	-
10 + 11	*TNFRSF13C* (BAFF-R) (*606269)	AD / AR	NM_052945	c.62C>G	p.Pro21Arg	Variation ID 341883 (likely benign)	16160919, 24406071	-

[$] The variants in *RNU4ATAC*, *KMT2A* and *IKZF1* are discussed in detail in the following chapters. AD: autosomal dominant, AR: autosomal recessive, XR: X-linked recessive, OMIM: Online Mendelian Inheritance in Man, dbSNP: Short Genetic Variations database, PMID: PubMed Identifier, CADD: Combined Annotation Dependent Depletion[25], MSC: Mutation Significance Cutoff[26].

4.3.3 Candidate gene approach

Based on the patients' phenotypes, selected genes were screened using either Sanger sequencing or NGS-based targeted resequencing: *NFKB1*, *NFKB2*, *SAP*, *XIAP*, *PIK3CD*, *PIK3R1*, *CTLA4*, *RAG1*, *RAG2*, *GATA2*, *BTK*, *IGLL1*, *IKZF1*, and *RNU4ATAC*. Sequencing covered all coding exons and intron-exon borders, as well as the unique noncoding exon of *RNU4ATAC* and intron 5 of *GATA2* that had been previously implicated in disease. A total of 162 genes were sequenced in the 70 index patients (minimum 2, maximum 5 and average 2.3 genes per patient). The candidate gene approach revealed four disease-causing mutations, meaning a clinical detection rate of 5.7% (4/70) (Figure 1, middle graph).

Patient 2 was a 22-month-old boy born to healthy non-consanguineous parents. He was identified with a known pathogenic missense variant in *BTK*, located on the X chromosome (Table 2). BTK is a key molecule in early B cell development and accounts for 85% of patients with agammaglobulinemia.[27] The here-identified mutation disrupts a highly conserved residue in the catalytic kinase domain of BTK, resulting in loss of protein function despite normal protein expression.[28,29] The patient presented with a typical laboratory phenotype of X-linked agammaglobulinemia, although he had an unusually mild clinical course (Table 3). The mother was an unaffected carrier of the mutant *BTK* allele.

Patient 3 had a history of atypical acute myeloid leukemia (AML) in childhood and post-AML perseverance of panhypogammaglobulinemia and B cell loss (Table 3). The latter initially gave her a tentative diagnosis of CVID. However, she also had reduced NK cells and monocytes (Table 3), which prompted us to sequence *GATA2*. GATA2 is a transcription factor important for early differentiation of hematopoietic cells in the bone marrow.[27] In case of pronounced antibody defects, GATA2 deficiency can show considerable phenotypical overlap with CVID.[5] Moreover, there had been previous reports on *GATA2* mutations in patients with a prior diagnosis of CVID.[30] Indeed, in patient 3, we detected a heterozygous *de novo* mutation in the regulatory region of intron 5 known to cause GATA2 deficiency (Table 2).[31,32]

In **patient 4**, an adult female with IPH and autoimmune manifestations (Table 3), we found a novel heterozygous missense variant in *NFKB1* (Table 2). The *NFKB1* variant was also detected in one of her two sons (11 years) and in her mother (70 years), although neither of them displayed clinical signs of immune deficiency. Interestingly, immunological laboratory assessment demonstrated IgA deficiency in the mutation-carrying son and IgG deficiency in the mother. The asymptomatic wild type son had no laboratory abnormalities. Autosomal dominant inheritance with incomplete penetrance as well as variable expressivity and variable age of onset had been previously described in *NFKB1* kindreds.[33] Altogether, the

here-reported novel heterozygous *NFKB1* variant was strongly suggestive to be disease-causing for the following reasons: *in silico* pathogenicity predictions for the variant were strong (Table 2), the index patient's phenotype was comparable to that of previously published *NFKB1* patients (Table 3)[33], and the variant segregated with the laboratory phenotype over three consecutive generations. Functional validation studies will be planned in the future.

Male index **patient 5** and his younger sister were both diagnosed with CVID in early childhood. With aging, however, syndromic features gradually became more conspicuous. In particular, the combination of antibody deficiency, spondyloepiphyseal dysplasia and retinal dystrophy in the male index patient raised a tentative diagnosis of Roifman syndrome (Table 3). This was later confirmed by the detection of compound heterozygous variants in the noncoding RNA gene *RNU4ATAC* (Table 2).[34] The unaffected parents each carried one of the *RNU4ATAC* variants. We elaborate on the siblings with *RNU4ATAC*-associated Roifman syndrome in **Chapter 5**.

4.3.4 Genome-wide approach using WES

WES was performed in 13 index patients: 9 patients with CVID, 2 patients with IPH, and 2 patients with syndromic PAD initially diagnosed as CVID. These patients were selected for WES because they fulfilled at least one of the following criteria: early disease onset, signs of immune dysregulation such as autoimmunity, positive family history of PAD, and/or consanguineous parents.[5] To facilitate variant analysis, WES was also performed in 27 (symptomatic and asymptomatic) family members of the 13 selected index patients.

With this approach, we found disease-causing variants in two out of 13 index patients (15%) and disease-modifying variants in four additional index patients (31%) (Figure 1, right graph).

Patient 6, one of monozygotic twins, was found to have a novel heterozygous variant in *KMT2A* causing Wiedemann-Steiner syndrome (Table 2).[35] The heterozygous *KMT2A* variant was also present in the other twin as well as in the mother. Similar to the siblings with *RNU4ATAC*-associated Roifman syndrome, the twins with *KMT2A*-associated Wiedemann-Steiner syndrome presented with a CVID-like phenotype in early childhood with syndromic features being initially less evident (Table 3). The mutation-carrying mother demonstrated congenital anomalies of the urogenital tract, recurrent respiratory tract infections, undetectable IgM serum levels and reduced switched memory B cells. This family is further discussed in **Chapter 5**.

We also identified a novel heterozygous variant in *IKZF1*, a gene only very recently associated with CVID (Table 2).[36] The variant was not only present in **patient 7** and her

younger sister who both had a CVID-like phenotype (Table 3), it was also shared by the asymptomatic mother. In **Chapter 6**, we provide evidence for the pathogenicity of the novel *IKZF1* variant found in this family.

In **4 of the 11 unsolved exomes** (i.e. exomes in which we did not find a disease-causing variant), we found **known disease-modifying variants**: 2 patients had variants in *TNFRSF13B* (encoding TACI) and 2 other patients in *TNFRSF13C* (encoding BAFF-R) (Figure 1, right graph). All four patients were clinically diagnosed with CVID (Table 3, patients 8-11). As explained in chapter 1, section 1.6.2.1, variants in *TNFRSF13B* and *TNFRSF13C* are by themselves not sufficient to establish disease but are considered to be modifier variants contributing to CVID.[5] **Patients 8 and 9** were heterozygous for the known p.Cys104Arg variant in *TNFRSF13B*. **Patient 10** and **patient 11** were heterozygous and homozygous, respectively, for the known p.Pro21Arg variant in *TNFRSF13C* (Table 2).[37-39] Thus far, no (likely) pathogenic variants could be identified in these four patients.

In **3 other unsolved exomes** we identified **novel candidate disease-causing genes**: *GTF3A*, *TAB1* and *RGL2* (Figure 1, right graph). Compound heterozygous variants in *GTF3A* were uncovered in two siblings manifesting a CVID-like phenotype. *TAB1* was found in a CVID/IPH pedigree showing autosomal dominant inheritance with incomplete penetrance and variable expressivity; and we recently identified a second family in our cohort with heterozygous mutations in *TAB1*. Finally, in a patient with IPH born to consanguineous parents, a homozygous variant in *RGL2* was revealed by combining WES with homozygosity mapping. In the latter patient, homozygous regions larger than 1 Mb were identified and used to facilitate WES variant filtering. For all three novel candidate disease-causing genes, there is strong *in silico* and genetic evidence of pathogenicity. Functional validation studies are currently ongoing, and are discussed in **Chapter 7** for *GTF3A*.

Finally, in the **4 remaining unsolved exomes** (4/11, 31%) (Figure 1, right graph), we did not detect known disease-modifying variants or novel candidate disease-causing genes. Sanger sequencing of insufficiently covered CVID-associated genes was also negative. Importantly, one of these exomes belonged to the patient with *RNU4ATAC*-associated Roifman syndrome described above (patient 5). When WES was performed in patient 5, no gene had been associated with this syndrome and the noncoding RNA gene *RNU4ATAC* was not identified by WES.[34]

Table 3. Clinical and immunological phenotype of the index patients with disease-causing or -modifying variants.

Patient (gene)	Sex	Prior clinical diagnosis	Age (years)	Disease onset	Main clinical features	IgG (g/L) †	IgG2 (g/L) †	IgG3 (g/L) †	IgA (g/L) †	IgM (g/L) †	Response to polysaccharide vaccine †	B cells (no./µL)	Switched memory B cells (%)	T cells (no./µL)	$CD4^+$ T cells (no./µL)	$CD8^+$ T cells (no./µL)	NK cells (no./µL)	Monocytes (no./µL)
1 (18q deletion)	M	Syndromic PAD	10	Infancy	Recurrent URTI and LRTI, recurrent GI infections, recurrent UTI, intellectual disability, short stature, congenital external auditory canal stenosis, Graves' disease	6.62 (2.9-10.2)	**0.24** (0.35-4.43)	0.67 (0.17-0.9)	**Undetec-table**	**Undetec-table**	**Insufficient**	**194** (200-1600)	**1** (10.9-30.4)	1720 (700-4200)	1290 (300-2000)	387 (300-1800)	215 (90-900)	**420** (700-1500)
2 (*BTK*)	M	Agamma-globulinemia	1.8	Infancy	Recurrent otitis media, adenitis colli, cellulitis	**0.3** (3.4-7.6)	**0.1** (0.38-2.4)	**0.019** (0.15-1.07)	**Undetec-table**	**Undetec-table**	**Absent**	**Undetec-table (<1%)**	**Undetec-table**	5280 (1400-8000)	2890 (900-5500)	2170 (400-2300)	278 (100-1400)	530 (400-1200)
3 (*GATA2*)	F	CVID	17	Childhood	Atypical AML in childhood, therapy-resistent warts on feet, mediastinal adenopathies	**4.3** (7.0-16.0)	1.91 (1.06-6.1)	0.231 (0.18-1.63)	**Undetec-table**	**Undetec-table**	**Insufficient**	**Undetec-table (<1%)**	**Undetec-table**	1030 (700-2100)	506 (300-1400)	517 (200-1200)	**66** (90-900)	**163** (500-1000)
4 (*NFKB1*)	F	IPH	37	Adulthood	Recurrent URTI and LRTI, recurrent vulvovaginal candidiasis, autoimmune optic neuropathy	**5.65** (7.0-16.0)	2.16 (1.5-6.4)	0.273 (0.2-1.1)	**0.69** (0.71-3.65)	0.96 (0.40-2.48)	Adequate	132 (100-500)	**7.3** (8.3-27.8)	939 (700-2100)	543 (300-1400)	352 (200-1200)	358 (90-600)	289 (247-757)
5 (*RNU4ATAC*)	M	Syndromic PAD (CVID)	14	Infancy	Recurrent URTI and LRTI, bronchiectasis, growth retardation, spondyloepiphyseal dysplasia, retinal dystrophy, dysmorphic facial features	**3.8** (4.7-9.3)	**0.53** (0.63-3.0)	**0.021** (0.13-1.26)	**0.3** (0.41-0.91)	**Undetec-table**	**Absent**	**36** (200-600)	9 (8.7-25.6)	940 (800-3500)	643 (400-2100)	274 (200-1200)	179 (70-1200)	910 (500-1000)
6 (*KMT2A*)	M	Syndromic PAD (CVID)	8	Infancy	Recurrent URTI and LRTI, bronchiectasis, mild developmental delay, intellectual disability, cardiovascular and urogenital anomalies, dysmorphic facial features	**2.8** (4.70-9.30)	**0.54** (0.63-3.0)	0.176 (0.13-1.26)	**0.3** (0.41-0.91)	**Undetec-table**	**Insufficient**	592 (200-1600)	**1** (10.9-30.4)	2580 (700-4200)	1640 (300-2000)	800 (300-1800)	244 (90-900)	570 (700-1500)
7 (*IKZF1*)	F	CVID	14	Early childhood	Recurrent URTI and LRTI, systemic onset JIA (monocyclic)	7 (4.70-11.9)	1.3 (0.98-4.8)	0.668 (0.15-1.49)	**Undetec-table**	**Undetec-table**	**Absent**	**17** (200-600)	**1.5** (8.7-25.6)	1370 (800-3500)	659 (400-2100)	575 (200-1200)	304 (70-1200)	730 (500-1000)
8 (*TNFRSF13B*, heterozygous)	F	CVID	14	Early childhood	Recurrent URTI, autoimmune cytopenias, psoriasis	**5.2** (7.0-16.0)	**0.72** (1.06-6.1)	0.218 (0.18-1.63)	**0.27** (0.71-3.65)	0.5 (0.40-2.48)	**Absent**	253 (200-600)	**6** (8.7-25.6)	1200 (800-3500)	664 (400-2100)	427 (200-1200)	126 (70-1200)	**260** (500-1000)
9 (*TNFRSF13B*, heterozygous)	M	CVID	16	Infancy	Recurrent URTI and LRTI, allergic asthma	**5.4** (7.0-16.0)	**0.635** (0.98-4.8)	0.227 (0.18-1.63)	**0.3** (0.41-0.91)	0.58 (0.4-1.8)	**Insufficient**	**706** (100-500)	**3** (8.3-27.8)	2270 (700-2100)	1010 (300-1400)	982 (200-1200)	92 (90-600)	530 (500-1000)
10 (*TNFRSF13C*, heterozygous)	F	CVID	14	Childhood	Recurrent URTI and LRTI, autoimmune cytopenias, lung granulomas, mediastinal lympadenopathies, mild hepatosplenomegaly, recurrent aphtous stomatitis, recurrent warts on hands and feet	**6.2** (7.0-16.0)	1.28 (1.06-6.1)	0.602 (0.18-1.63)	**0.4** (0.71-3.65)	0.9 (0.40-2.48)	**Insufficient**	**174** (200-600)	**2** (8.7-25.6)	**496** (800-3500)	**306** (400-2100)	**132** (200-1200)	149 (70-1200)	**360** (500-1000)
11 (*TNFRSF13C*, homozygous)	M	CVID	72	Late adulthood	Recurrent URTI and LRTI, bronchiectasis, autoimmune cytopenias, enteropathy	**3.6** (7.0-16.0)	**1.08** (1.5-6.4)	**0.156** (0.2-1.1)	**Undetec-table**	**Undetec-table**	**Insufficient**	**Undetec-table (<1%)**	**Undetec-table**	**590** (700-2100)	**174** (300-1400)	50 (10-39)	234 (90-600)	590 (307-802)

Age-based reference values are given in parentheses. Abnormal lab values are indicated in bold. Patients 5, 6 and 7 are discussed in detail in the following chapters. Disease onset: infancy (0–2 years), early childhood (3–8 years), childhood (9–17 years), adulthood (18–50 years), late adulthood (>50 years). † Measured at time of diagnosis. AML: acute myeloid leukemia, CVID: common variable immunodeficiency, GI: gastrointestinal, IPH: idiopathic primary hypogammaglobulinemia, JIA: juvenile idiopathic arthritis, LRTI: lower respiratory tract infections, PAD: primary antibody deficiency, URTI: upper respiratory tract infections, UTI: urinary tract infections.

4.3.5 Summary

Together, disease-causing mutations were, so far, found in 7 out of 70 index patients (10% of the cohort): two patients clinically diagnosed with CVID, one with IPH, one with agammaglobinemia and three with syndromic PAD (Figure 2A). Besides unaffected carriers of recessive alleles, the disease-causing mutations were also identified in four symptomatic family members as well as in three family members without PAD-associated clinical features (Figure 2B). In four additional index patients, WES revealed variants in *TNFRSF13B* (encoding TACI) or *TNFRSF13C* (encoding BAFF-R), considered to be modifier genes contributing to CVID.[5] Furthermore, we discovered three novel candidate disease genes by means of WES, for which functional validation is currently ongoing (data not shown).

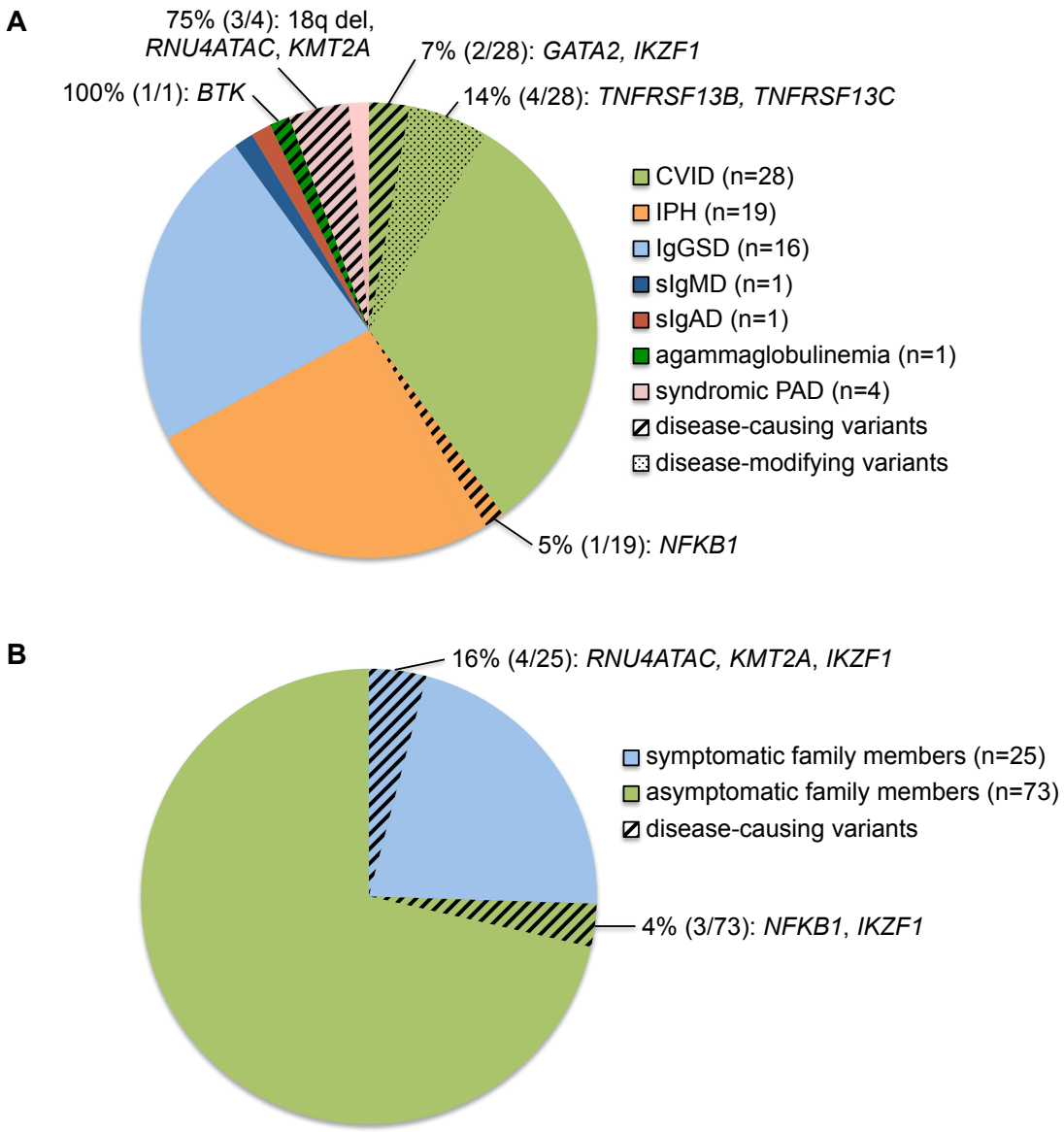


Figure 2. Overview of the Ghent PAD cohort and the identified disease-associated variants. (A) The Ghent PAD cohort of 70 index patients was divided over 7 different clinical diagnoses. Genes in which disease-associated variants were identified, are indicated on the graph per clinical diagnosis. PAD: primary antibody deficiency, CVID: common variable immunodeficiency, IPH: idiopathic primary hypogammaglobulinemia, IgGSD: IgG subclass deficiency, sIgMD: selective IgM deficiency, sIgAD: selective IgA deficiency, 18q del: 18q deletion. **(B)** 98 family members of the index patients were included in the study, of which 25 were symptomatic and diagnosed with a form of PAD. Relatives in which disease-causing variants were identified, are indicated on the graph. Unaffected carriers of recessive alleles were not taken into account.

4.4 Proposed strategy for genetic testing in CVID and related disorders

At the start of this doctoral research project, the technologies used to obtain a genetic diagnosis in PID patients had come at a turning point. Up to 2012-2013, the most commonly used method was Sanger sequencing of one or more candidate genes based on the patient's phenotype. However, this was a very time consuming and expensive approach with relatively low success rates.[40] Since then, the implementation of NGS technologies in genetics labs worldwide has drastically changed the PID landscape (see chapter 1, section 1.2.1.1).[2] In brief, NGS-based methods as targeted resequencing of gene panels and WES allowed for large numbers of genes to be screened simultaneously, increasing the diagnostic success rate in PID patients.[2,40] In parallel, the use of NGS technologies in research setting has greatly facilitated the discovery of novel PID-causing genes, which allows for more patients to receive a genetic diagnosis.[2] However, with the discovery of numereous new genes over the past few years, it has become clear that many monogenic PIDs do not obey Mendelian laws of inheritance, showing variable expressivity and even incomplete penetrance. For CVID and CVID-like disorders, the situation is even more complex because, since 2015-2016, it is increasingly believed that the majority of these patients have a polygenic or multifactorial rather than a monogenic etiology (see chapter 1, section 1.6).[5] Altogether, this makes it difficult to develop guidelines for the genetic workup in CVID(-like) patients, both from a clinical and research point of view.

Based on our experience from the Ghent cohort, we **propose a workflow for genetic testing in patients with CVID and related antibody disorders in whom a monogenic cause is suspected**, applicable in both a clinical and research setting (Figure 3). As mentioned above, a monogenic cause is more probable in case of early disease onset, features of immune dysregulation such as autoimmunity/inflammation, a positive family history, and/or consanguinity.[5,6] First, we recommend low-cost screening of CNVs by means of array CGH in all patients (Figure 3). Although disease-causing CNVs have, so far, not often been detected in CVID(-like) patients, they should be excluded. Homozygosity mapping is the preferred approach in consanguineous families, and our SNP chip array-based method can also be used for CNV analysis (Figure 3). Next, targeted sequencing of a selected set of genes can be useful if the patient's phenotype is typical for a limited number of specific genotypes (Figure 3).[2] This is, however, rarely the case. NGS-based gene panels enable us to screen all PID-causing genes at the same time, but these are very hard to keep up to date because of the unseen pace at which new genes are discovered (Figure 3).[2] Furthermore, gene panels limit the discovery of new disease-causing genes making them less attractive for researchers. Therefore, WES is currently the most cost-effective approach both from a

clinical and research point of view (Figure 3).[2] Whole genome sequencing (WGS) is not yet widely available, but has several technical advantages over WES and also allows for more reliable identification of structural variants including CNVs.[2] As the costs for WGS are going down, this technique will most likely replace the combined array CGH - WES approach in the near future (Figure 3).[2]

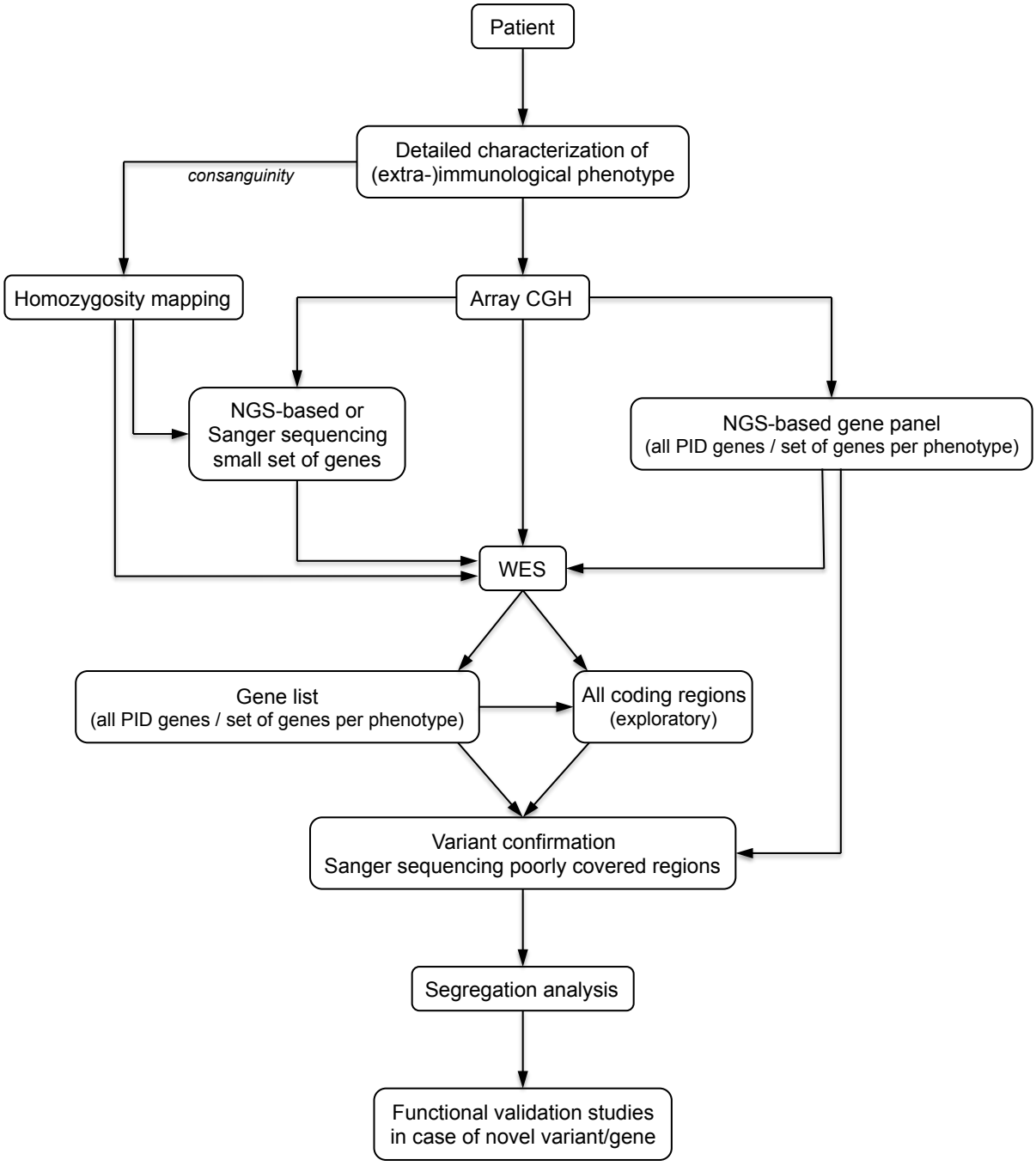


Figure 3. Flowchart for genetic testing in patients with CVID and related antibody disorders in whom a monogenic cause is suspected. Microarray-based comparative genomic hybridization (array CGH) is recommended as a first, low-cost, step to screen for copy number variations (CNVs). In patients born to consanguineous parents, homozygosity mapping (also called autozygosity mapping or identity-by-descent (IBD) mapping) is preferred. If generated using SNP chip arrays, IBD data can also be used for analysis of CNVs, omitting the need for array CGH. If a genetic diagnosis cannot be obtained by a specific approach, proceed to the next step in the flowchart up until whole exome sequencing (WES). WES data can be filtered against gene lists to narrow down the number of variants. Alternatively, all variants can be explored. Variants identified with NGS-based techniques are usually confirmed using Sanger sequencing. Novel variants or genes need to be functionally validated to prove their causality. Note that this flowchart can be extended to all patients with primary immunodeficiencies (PIDs). NGS: next-generation sequencing.

4.5 Discussion

So far, genetic testing of the Ghent PAD cohort rendered a genetic diagnosis in 7 out of 70 index patients (10%). Three of those 7 patients had a syndromic PAD, suggesting that the presence of extra-immunological features facilitates the identification of the underlying genetic defect.[14] In our cohort, a genetic diagnosis could be given to 3 of the 4 syndromic PAD patients (75%): 18q deletion syndrome, *RNU4ATAC*-associated Roifman syndrome and *KMT2A*-associated Wiedemann-Steiner syndrome. Furthermore, our cohort counted one male patient with agammaglobulinemia. Sequencing of the X-linked gene *BTK*, the most obvious candidate gene in that patient, provided a definitive molecular diagnosis.

Ninety percent of the Ghent PAD cohort (n=63) consisted of patients clinically diagnosed with CVID or the related disorders IPH and IgGSD. Within that group, disease-causing variants could only be discerned in 3 patients (~ 5%), namely heterozygous variants in *GATA2*, *NFKB1*, and *IKZF1*. This was markedly lower than what was reported in literature, where disease-causing variants can be identified in up to 15% of patients.[6,41] However, in our cohort, WES has so far only been performed in a small number of patients and future research is likely to recognize disease-causing variants in additional patients. Moreover, CVID(-like) patients reported in literature are often preselected based on more severe phenotypes, which increases the probability of detecting a monogenic cause.[5,6]

The past years, an NGS-driven revolution has taken place in the field of PID that resulted in the discovery of numerous novel disease genes and entities.[2] Unfortunately, for many disease entities, a phenotype-genotype correlation is virtually non-existent.[2] This was also noted in our cohort: sequencing of selected genes based on patients' phenotypes had a low yield of about 5.7%. In contrast, WES provided a conclusive genetic diagnosis in 15% of tested patients. Furthermore, it is clear that non-Mendelian inheritance patterns such as variable expressivity and incomplete penetrance are the rule rather than the exception in the majority of PIDs. In the Ghent PAD cohort, this was illustrated by the presence of disease-causing mutations in 3 out of 73 asymptomatic family members (4%), not including unaffected carriers of recessive alleles.

Based on our findings, we concur with the generally accepted notion that, in PID patients, targeted testing of small sets of genes is becoming obsolete.[2] This is especially true for CVID and related disorders, which show considerable phenotypical overlap with many other PID entities.[5] Hence, WES is currently the most cost-effective approach for diagnostic as well as research purposes, and we expect that in the near future WGS will become the preferred method.[2]

References

1. Strachan T, Read A. Human Molecular Genetics. 4th ed. Garland Science, Taylor & Francis Group, LLC; 2011.
2. Meyts I, Bosch B, Bolze A, et al. Exome and genome sequencing for inborn errors of immunity. J Allergy Clin Immunol. 2016;138(4):957-69.
3. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977;74(12):5463-7.
4. Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. Nat Rev Genet. 2016;17(6):333-51.
5. Bogaert DJ, Dullaers M, Lambrecht BN, Vermaelen KY, De Baere E, Haerynck F. Genes associated with common variable immunodeficiency: one diagnosis to rule them all? J Med Genet. 2016;53(9):575-90.
6. Maffucci P, Filion CA, Boisson B, et al. Genetic Diagnosis Using Whole Exome Sequencing in Common Variable Immunodeficiency. Front Immunol. 2016;7:220.
7. Bogaert DJ, De Bruyne M, Debacker V, et al. The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. Haematologica. 2017;102(1):192-202.
8. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol. 1999;93(3):190-7.
9. Driessen GJ, van Zelm MC, van Hagen PM, et al. B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood. 2011;118(26):6814-23.
10. Pan Q, Hammarstrom L. Molecular basis of IgG subclass deficiency. Immunol Rev. 2000;178:99-110.
11. Goldstein MF, Goldstein AL, Dunsky EH, Dvorin DJ, Belecanech GA, Shamir K. Selective IgM immunodeficiency: retrospective analysis of 36 adult patients with review of the literature. Ann Allergy Asthma Immunol. 2006;97(6):717-30.
12. Yel L. Selective IgA deficiency. J Clin Immunol. 2010;30(1):10-6.
13. Conley ME, Broides A, Hernandez-Trujillo V, et al. Genetic analysis of patients with defects in early B-cell development. Immunol Rev. 2005;203:216-34.
14. Ming JE, Stiehm ER. Genetic syndromic immunodeficiencies with antibody defects. Immunol Allergy Clin North Am. 2008;28(4):715-36, vii.
15. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 2007;35(Web Server issue):W71-4.
16. De Leeneer K, Hellemans J, Steyaert W, et al. Flexible, scalable, and efficient targeted resequencing on a benchtop sequencer for variant detection in clinical practice. Hum Mutat. 2015;36(3):379-87.
17. Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet. 2010;11(1):31-46.
18. Meienberg J, Zerjavic K, Keller I, et al. New insights into the performance of human whole-exome capture platforms. Nucleic Acids Res. 2015;43(11):e76.
19. Coppieters F, Van Schil K, Bauwens M, et al. Identity-by-descent-guided mutation analysis and exome sequencing in consanguineous families reveals unusual clinical and molecular findings in retinal dystrophy. Genet Med. 2014;16(9):671-80.
20. Menten B, Pattyn F, De Preter K, et al. arrayCGHbase: an analysis platform for comparative genomic hybridization microarrays. BMC Bioinformatics. 2005;6:124.
21. Alkuraya FS. Homozygosity mapping: one more tool in the clinical geneticist's toolbox. Genet Med. 2010;12(4):236-9.
22. Feenstra I, Vissers LE, Orsel M, et al. Genotype-phenotype mapping of chromosome 18q deletions by high-resolution array CGH: an update of the phenotypic map. Am J Med Genet A. 2007;143A(16):1858-67.
23. Cody JD, Ghidoni PD, DuPont BR, et al. Congenital anomalies and anthropometry of 42 individuals with deletions of chromosome 18q. Am J Med Genet. 1999;85(5):455-62.

24. Aghamohammadi A, Mohammadi J, Parvaneh N, et al. Progression of selective IgA deficiency to common variable immunodeficiency. Int Arch Allergy Immunol. 2008;147(2):87-92.
25. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet. 2014;46:310-5.
26. Itan Y, Shang L, Boisson B, et al. The mutation significance cutoff: gene-level thresholds for variant predictions. Nat Methods. 2016;13(2):109-10.
27. Durandy A, Kracker S, Fischer A. Primary antibody deficiencies. Nat Rev Immunol. 2013;13(7):519-33.
28. Hagemann TL, Chen Y, Rosen FS, Kwan SP. Genomic organization of the Btk gene and exon scanning for mutations in patients with X-linked agammaglobulinemia. Hum Mol Genet. 1994;3(10):1743-9.
29. Zhu Q, Zhang M, Winkelstein J, Chen SH, Ochs HD. Unique mutations of Bruton's tyrosine kinase in fourteen unrelated X-linked agammaglobulinemia families. Hum Mol Genet. 1994;3(10):1899-900.
30. Chou J, Lutskiy M, Tsitsikov E, Notarangelo LD, Geha RS, Dioun A. Presence of hypogammaglobulinemia and abnormal antibody responses in GATA2 deficiency. J Allergy Clin Immunol. 2014;134(1):223-6.
31. Ganapathi KA, Townsley DM, Hsu AP, et al. GATA2 deficiency-associated bone marrow disorder differs from idiopathic aplastic anemia. Blood. 2015;125:56-70.
32. Hsu AP, Johnson KD, Falcone EL, et al. GATA2 haploinsufficiency caused by mutations in a conserved intronic element leads to MonoMAC syndrome. Blood. 2013;121:3830-7, S1-7.
33. Fliegauf M, V LB, Frede N, et al. Haploinsufficiency of the NF-kappaB1 Subunit p50 in Common Variable Immunodeficiency. Am J Hum Genet. 2015;97(3):389-403.
34. Merico D, Roifman M, Braunschweig U, et al. Compound heterozygous mutations in the noncoding RNU4ATAC cause Roifman Syndrome by disrupting minor intron splicing. Nat Commun. 2015;6:8718.
35. Stellacci E, Onesimo R, Bruselles A, et al. Congenital immunodeficiency in an individual with Wiedemann-Steiner syndrome due to a novel missense mutation in KMT2A. Am J Med Genet A. 2016;170(9):2389-93.
36. Kuehn HS, Boisson B, Cunningham-Rundles C, et al. Loss of B Cells in Patients with Heterozygous Mutations in IKAROS. N Engl J Med. 2016;374(11):1032-43.
37. Castigli E, Wilson SA, Garibyan L, et al. TACI is mutant in common variable immunodeficiency and IgA deficiency. Nat Genet. 2005;37(8):829-34.
38. Salzer U, Chapel HM, Webster AD, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. Nat Genet. 2005;37:820-8.
39. Pieper K, Rizzi M, Speletas M, et al. A common single nucleotide polymorphism impairs B-cell activating factor receptor's multimerization, contributing to common variable immunodeficiency. J Allergy Clin Immunol. 2014;133(4):1222-5.
40. Stoddard JL, Niemela JE, Fleisher TA, Rosenzweig SD. Targeted NGS: A Cost-Effective Approach to Molecular Diagnosis of PIDs. Front Immunol. 2014;5:531.
41. Gallo V, Dotta L, Giardino G, et al. Diagnostics of Primary Immunodeficiencies through Next-Generation Sequencing. Front Immunol. 2016;7:466.

Chapter 5

Early-onset primary antibody deficiency resembling common variable immunodeficiency challenges the diagnosis of Wiedemann-Steiner and Roifman syndromes

5 EARLY-ONSET PRIMARY ANTIBODY DEFICIENCY RESEMBLING COMMON VARIABLE IMMUNODEFICIENCY CHALLENGES THE DIAGNOSIS OF WIEDEMANN-STEINER AND ROIFMAN SYNDROMES

Delfien J. Bogaert, MD[1,2,3,4], Melissa Dullaers, PhD[1,4,5], Hye Sun Kuehn, PhD[6], Bart P. Leroy, MD, PhD[3,7,8], Julie E. Niemela, MSc[6], Hans De Wilde, MD[9], Sarah De Schryver, MD[10], Marieke De Bruyne, MSc[3], Frauke Coppieters, PhD[3], Bart N. Lambrecht, MD, PhD[4,5,11], Frans De Baets, MD, PhD[2], Sergio D. Rosenzweig, MD, PhD[6], Elfride De Baere, MD, PhD[3,*] and Filomeen Haerynck, MD, PhD[1,2,*]

[1]Clinical Immunology Research Lab, Department of Pulmonary Medicine, Centre for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, Belgium; [2]Department of Paediatric Immunology and Pulmonology, Centre for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, Belgium; [3]Center for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium; [4]Laboratory of Immunoregulation, VIB Inflammation Research Centre, Ghent, Belgium; [5]Department of Internal Medicine, Ghent University, Ghent, Belgium; [6]Immunology Service, Department of Laboratory Medicine, NIH Clinical Centre, National Institutes of Health, Bethesda, MD, USA; [7]Department of Ophthalmology, Ghent University Hospital, Ghent, Belgium; [8]Division of Ophthalmology, The Children's Hospital of Philadelphia, Philadelphia, USA; [9]Department of Paediatric Cardiology, Ghent University Hospital, Ghent, Belgium; [10]Department of Paediatric Allergy and Immunology, Montreal Children's Hospital, Montreal, QC, Canada; [11]Department of Pulmonology, Ghent University Hospital, Ghent, Belgium; [*]EDB and FH contributed equally to this work.

Scientific Reports 2017;7(1):3702. doi: 10.1038/s41598-017-02434-4.

www.nature.com/scientificreports

SCIENTIFIC REPORTS

OPEN

Early-onset primary antibody deficiency resembling common variable immunodeficiency challenges the diagnosis of Wiedeman-Steiner and Roifman syndromes

Delfien J. Bogaert[1,2,3,4], Melissa Dullaers[1,4,5], Hye Sun Kuehn[6], Bart P. Leroy[3,7,8], Julie E. Niemela[6], Hans De Wilde[9], Sarah De Schryver[10], Marieke De Bruyne[3], Frauke Coppieters[3], Bart N. Lambrecht[4,5,11], Frans De Baets[2], Sergio D. Rosenzweig[6], Elfride De Baere[3] & Filomeen Haerynck[1,2]

Syndromic primary immunodeficiencies are rare genetic disorders that affect both the immune system and other organ systems. More often, the immune defect is not the major clinical problem and is sometimes only recognized after a diagnosis has been made based on extra-immunological abnormalities. Here, we report two sibling pairs with syndromic primary immunodeficiencies that exceptionally presented with a phenotype resembling early-onset common variable immunodeficiency, while extra-immunological characteristics were not apparent at that time. Additional features not typically associated with common variable immunodeficiency were diagnosed only later, including skeletal and organ anomalies and mild facial dysmorphism. Whole exome sequencing revealed *KMT2A*-associated Wiedemann-Steiner syndrome in one sibling pair and their mother. In the other sibling pair, targeted testing of the known disease gene for Roifman syndrome (*RNU4ATAC*) provided a definite diagnosis. With this study, we underline the importance of an early-stage and thorough genetic assessment in paediatric patients with a common variable immunodeficiency phenotype, to establish a conclusive diagnosis and guide patient management. In addition, this study extends the mutational and immunophenotypical spectrum of Wiedemann-Steiner and Roifman syndromes and highlights potential directions for future pathophysiological research.

[1]Clinical Immunology Research Lab, Department of Pulmonary Medicine, Centre for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, Belgium. [2]Department of Paediatric Immunology and Pulmonology, Centre for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, Belgium. [3]Center for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium. [4]Laboratory of Immunoregulation, VIB Inflammation Research Centre, Ghent, Belgium. [5]Department of Internal Medicine, Ghent University, Ghent, Belgium. [6]Immunology Service, Department of Laboratory Medicine, NIH Clinical Centre, National Institutes of Health, Bethesda, MD, USA. [7]Department of Ophthalmology, Ghent University Hospital, Ghent, Belgium. [8]Division of Ophthalmology, The Children's Hospital of Philadelphia, Philadelphia, USA. [9]Department of Paediatric Cardiology, Ghent University Hospital, Ghent, Belgium. [10]Department of Paediatric Allergy and Immunology, Montreal Children's Hospital, Montreal, QC, Canada. [11]Department of Pulmonology, Ghent University Hospital, Ghent, Belgium. Elfride De Baere and Filomeen Haerynck contributed equally to this work. Correspondence and requests for materials should be addressed to F.H. (email: Filomeen.Haerynck@uzgent.be)

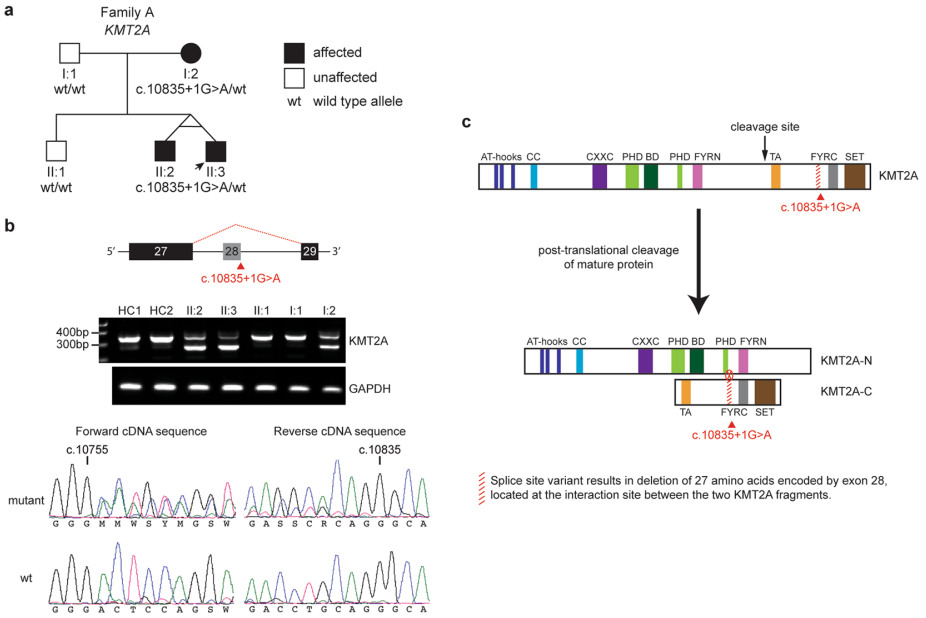


Figure 1. Family A with *KMT2A*-associated Wiedemann-Steiner syndrome (WSS). (**a**) Pedigree of family A. (**b**) Skipping of *KMT2A* exon 28. Gel electrophoresis of the *KMT2A* cDNA region containing exon 28 revealed a second shorter transcript in the three affected individuals. HC1 and HC2 represent two healthy controls; *GAPDH* was used as reference target. In-frame deletion of exon 28 was confirmed by cDNA sequencing; c.10755 and c.10835 indicate the start respectively stop position of exon 28. (**c**) KMT2A protein domains (adapted from ref. 9). KMT2A is cleaved in an N-terminal (KMT2A-N) and C-terminal (KMT2A-C) fragment, which form a non-covalently associated complex. Deletion of the amino acids encoded by exon 28 may disrupt the interaction site between the two fragments.

Common variable immunodeficiency (CVID) is one of the most frequently diagnosed primary immunodeficiencies (PIDs), and is defined as decreased serum immunoglobulin (Ig) G, decreased IgA and/or IgM, poor antibody responses to vaccines, and exclusion of other causes of hypogammaglobulinemia[1]. Patients commonly experience recurrent (sinopulmonary) infections and features of immune dysregulation such as autoimmunity[1,2]. About 25% of CVID patients are diagnosed in childhood[3]. To rule out transient hypogammaglobulinemia of infancy, in which Ig levels spontaneously resolve mostly by the age of two to four years, a definite diagnosis of CVID should not be given before the age of four years[1].

Here, we report novel familial cases of Wiedemann-Steiner syndrome (WSS) and Roifman syndrome (RS) that were initially categorized as early-onset CVID. WSS and RS are rare syndromic PIDs, affecting the immune system as well as other organ systems[4–6]. Although there is considerable phenotypic heterogeneity in both syndromes, hallmark extra-immunological features are generally evident very early in life[7,8]. WSS is typically characterized by *hypertrichosis cubiti*, growth retardation, developmental delay and facial dysmorphism, and is caused by heterozygous mutations in *lysine methyltransferase 2 A* (*KMT2A*)[7,9–14]. *KMT2A* (also called *mixed-lineage leukemia*, *MLL*) encodes a histone methyltransferase involved in regulating chromatin-mediated transcription and is a frequent target of chromosomal rearrangements in childhood leukemia[9,15]. WSS has only been recently associated with primary antibody deficiency[7]. RS, on the other hand, is commonly featured by antibody deficiency as well as growth retardation, spondyloepiphyseal dysplasia and retinal dystrophy[8,16]. Biallelic mutations in *RNU4ATAC*, a noncoding small nuclear RNA (snRNA) gene, were recently identified as a cause of RS[8]. U4atac snRNA is an important component of the minor spliceosome required for minor intron splicing[8].

This report aspires to increase awareness among immunologists and geneticists that a CVID phenotype can be the principal presentation of WSS and RS in early childhood, which is exceptional and has not been previously reported. The prior diagnosis of early-onset CVID diverted attention away from the initially less evident extra-immunological features, which significantly delayed identification of the underlying syndromic disorders. Additionally, in both families we identified mutations that have not been previously associated with disease. We aimed to provide insight as to how these mutations are disease-causing. Finally, we expand the immunophenotypical spectrum of WSS and RS, which could support future mechanistic research.

Results

An early-onset CVID phenotype in two unrelated sibling pairs. This study reports on two unrelated sibling pairs with recurrent respiratory tract infections and antibody deficiency categorized as CVID in early childhood. The family A monozygotic twin boys (Fig. 1a, II:2 and II:3) were born prematurely at 34 weeks

gestational age to non-consanguineous, Belgian parents and are currently 11 years old. One of them (II:3) was born with bilateral inguinal hernia and hypospadias, which were attributed to his premature birth. From the first year of life, the twin boys suffered from recurrent upper and lower respiratory tract infections, often requiring antibiotics. At 2 months of age, patient II:3 developed severe pneumonia with respiratory arrest and heart failure. The latter led to the recognition of a patent *ductus arteriosus*, which was surgically ligated shortly thereafter. The postoperative course was complicated by severe respiratory distress requiring ventilation and systemic corticosteroids. Upon immunological evaluation, both patients II:2 and II:3 demonstrated panhypogammaglobulinemia, poor antibody responses to Pneumococcal polysaccharide vaccine, increased naive B cells, and very low memory B cells (Table 1). Additionally, both patients showed evidence of mild bronchiectasis on high-resolution computed tomography (HRCT) scan at 3.5 years of age.

The family B brother and sister pair (Fig. 2a, II:1 and II:2) were born to healthy, non-consanguineous, Belgian parents and are currently 17 and 14 years of age respectively. They had recurrent upper and lower respiratory tract infections since the age of 18 months (II:1) and 3 years (II:2). The girl (II:2) also had recurrent gastroenteritis in early childhood and diffuse atopic eczema since infancy. Similar to the family A sibling pair, they had panhypogammaglobulinemia and poor global antibody responses to Pneumococcal polysaccharide vaccine (Table 2). Serotype-specific Pneumococcal antibody responses were not evaluated. In contrast to the family A twins, the family B siblings demonstrated severe B cell lymphopenia with normal switched memory B cell percentages and increased $CD21^{low}$ B cell levels (Table 2). HRCT scan in patient II:1 at the age of 7.5 years displayed marked bronchiectasis, whereas his sister (II:2) only showed discrete bronchiectasis at a similar age.

In both the family A and B sibling pairs, the clinical presentation and laboratory findings in the first years of life were reminiscent of a CVID phenotype. All patients are currently doing well under regular Ig replacement therapy, antiflogistic maintenance treatment with azithromycin and intermittent inhaled corticosteroids and/or short-acting beta-agonists therapy.

Extra-immunological features raised suspicion of syndromic PID. Although both sibling pairs first presented with a phenotype resembling CVID, with time they gradually demonstrated additional clinical features not typically associated with CVID (Tables 3 and 4). The family A twin boys developed a third degree atrioventricular block for which a pacemaker was implanted at 5 (II:3) and 6.5 (II:2) years of age respectively. In the following years, the boys showed increasing evidence of mild intellectual disability. In retrospect, patient II:3 had signs of mild developmental delay during the first years of life. Both twins also demonstrated poor weight gain and growth retardation, albeit to a limited extent. Around the age of 9 years, dysmorphic facial features became more conspicuous (Table 3). Interestingly, the twins' mother (I:2) had congenital urogenital tract anomalies consisting of a unicornuate uterus and a unique left ovary, fallopian tube and kidney. Moreover, since childhood, she had suffered from right unilateral sensorineural hearing loss as well as recurrent sinusitis and bronchitis frequently requiring antibiotics. At that time, genetic or immunological testing had never been performed in the mother as she deemed herself to be in good general health. The twins' older brother (II:1) and father (I:1) had an uneventful medical history.

Analogously, the boy in family B (II:1) initially displayed subtle syndromic features, such as mild growth retardation, that appeared more pronounced over time. At the age of seven years, diverse skeletal abnormalities including spondyloepiphyseal dysplasia were detected (Table 4). At the same age, he was also found to have slowly progressive retinal dystrophy (Fig. 2b, Panels I-III) with decreased rod function but near-normal cone function on full-field flash electroretinography. Antibody deficiency in combination with skeletal and ophthalmological features led to the clinical suspicion of RS in the boy. However, his sister (II:2) had no radiographic sings of spondyloepiphyseal or hip dysplasia nor retinal dystrophy. Moreover, RS was originally presumed to be an X-linked recessive condition, although no causal gene had been identified[16]. When the family B girl (II:2) was about 9 years old, Gray *et al*. reported the first female patient with RS, having a skewed X-inactivation and a milder phenotype than her affected brother[17]. Subsequently, we hypothesized that patient II:2 might be a manifesting heterozygote of RS, which could be compatible with her milder extra-immunological phenotype at that time.

Cytogenetic and cytogenomic analyses were negative in both sibling pairs. In the family A twins, conventional G-banding karyotype, fluorescent *in situ* hybridization for region 22q11.2 and subtelomeric screening were normal. Furthermore, microarray-based comparative genomic hybridisation analysis in both sibling pairs did not demonstrate copy number variations.

Whole exome sequencing (WES) uncovers WSS in family A. Since no specific genetic syndrome was suspected in family A, WES was performed in patient II:2 and both parents. This revealed a heterozygous splice site variant in *KMT2A* (NM_001197104:c.10835 + 1 G > A), present in the twins (II:2, II:3) as well as in the mother (I:2) (Fig. 1a). The variant is not reported in public or in-house databases. The *KMT2A* nucleotide substitution is situated in the splice donor site of intron 28. *In silico* splicing prediction tools suggested complete loss of the splice donor site resulting in exon 28 skipping and an in-frame deletion of 81 bp, which was confirmed by analyses on cDNA derived from patients' PBMCs (Fig. 1b). Mature KMT2A protein is physiologically cleaved in an N-terminal (KMT2A-N) and C-terminal (KMT2A-C) fragment, which together form a non-covalently associated complex (Fig. 1c)[15,18]. Complex formation is necessary for stability and subnuclear localization of the protein[18]. The amino acids encoded by exon 28 are part of the interaction site between KMT2A-N and KMT2A-C (Fig. 1c)[15]. It has been shown that disrupting the interaction between the two fragments causes degradation of the KMT2A-N fragment and loss of protein function[18]. The KMT2A-N fragment was only very weakly detectable by western blot on PBMC lysates, however, and could therefore not be reliably interpreted (data not shown). Subsequent investigations in the mother demonstrated mild intellectual disability, undetectable serum IgM, and

	Patient II:2		Patient II:3		Patient I:2	
	Value	Reference range	Value	Reference range	Value	Reference range
White blood cells	*Age: 8 years*		*Age: 8 years*		*Age: 45 years*	
Total leukocytes (no./μL)	11930	6000–14000	7340	6000–14000	7210	3650–9300
Neutrophils (no./μL)	6980	2000–8000	3150	2000–8000	3450	1573–6100
Lymphocytes (no./μL)	3990	1500–7500	3480	1500–7500	2870	1133–3105
CD3+ T cells (no./μL)	2630	700–4200	2580	700–4200	2240	700–2100
CD3+ CD4+ T helper cells (no./μL)	1600	300–2000	1640	300–2000	1120	300–1400
CD45RA + naive CD4 + T cells (%)	82	46–77†	82	46–77†	44	NA
CD45RO+ memory CD4+ T cells (%)	12	13–30†	10	13–30†	50	NA
CD3+ CD8+ T cytotoxic cells (no./μL)	838	300–1800	800	300–1800	1060	200–1200
CD45RA+ naive CD8+ T cells (%)	80	63–92†	69	63–92†	26	NA
CD45RO+ memory CD8+ T cells (%)	12	4–21†	10	4–21†	73	NA
CD19+ B cells (no./μL)	798	200–1600	592	200–1600	287	100–500
IgD + CD27- naive B cells (%)	96	47.3–77.0‡	94	47.3–77.0‡	88	48.4–79.7‡
CD24 + + CD38++ transitional B cells (%)	19	4.6–8.3‡	10	4.6–8.3‡	14	0.9–5.7‡
IgD − CD27+ switched memory B cells (%)	1	10.9–30.4‡	1	10.9–30.4‡	6	8.3–27.8‡
IgD + CD27+ marginal zone B cells (%)	2	5.2–20.4‡	2	5.2–20.4‡	3	7.0–23.8‡
$CD21^{low}$ $CD38^{low}$ B cells (%)	2	2.3–10.0‡	3	2.3–10.0‡	2	1.6–10.0‡
CD3 − CD56 + CD16+ NK cells (no./μL)	479	90–900	244	90–900	344	90–600
Monocytes (no./μL)	690	700–1500	570	700–1500	780	247–757
Eosinophils (no./μL)	220	200–500	100	200–500	80	28–273
Basophils (no./μL)	30	10–100	20	10–100	10	6–50
Immunoglobulins*	*Age: 7 years*		*Age: 3 years*		*Age: 45 years*	
IgG (g/L)	3.6	4.70–10.5	2.8	4.70–9.30	7.6	7.0–16.0
IgG2 (g/L)	1.13	0.85–4.10	0.54	0.63–3.0	3.03	1.50–6.40
IgG3 (g/L)	0.265	0.13–1.42	0.176	0.13–1.26	0.307	0.20–1.10
IgM (g/L)	<0.2	0.27–0.63	<0.2	0.27–0.57	<0.18	0.40–2.48
IgA (g/L)	0.3	0.50–1.41	0.3	0.41–0.91	2.35	0.71–3.65
IgE (kU/L)	<4.4	0–90	<4.4	0–60	NA	
Specific antibody responses*	*Age: 7 years*		*Age: 3 years*		*Age: 45 years*	
S. pneumoniae polysaccharide IgG (Lab U)	NA		9	≥11: immune	NA	
S. pneumoniae polysaccharide IgG: specific IgG response to 3 serotypes (8, 9 N, 15B)	Insufficient antibody response	2x titer increase for at least 2 out of 3 serotypes	NA		Good antibody response	2x titer increase for at least 2 out of 3 serotypes
Tetanus IgG (IU/mL)	0.01	≥0.01: immune	0.03	≥0.01: immune	0.50	≥0.01: immune
Rubella IgG (IU/mL)	12	>10: immune	44	>10: immune	NA	
Measles IgG (mIU/mL)	350	>300: immune	1200	>300: immune	NA	
Mumps IgG (Lab U/mL)	270	>500: immune	540	>500: immune	NA	
Varicella Zoster IgG (mIU/mL)	620	>100: immune	1400	>100: immune	NA	
Lymphocyte proliferation assay	*Age: 3 years*		*Age: 3 years*			
Response to Concanavalin A	Normal	Compared to control	Normal	Compared to control	NA	
Response to Phytohemagglutinin	Normal	Compared to control	Normal	Compared to control	NA	
Response to Tetanus toxoid	Normal	Compared to control	Moderately reduced	Compared to control	NA	

Table 1. Routine immunological laboratory results of the family A patients with Wiedemann-Steiner syndrome. The most recent, comprehensive and representative laboratory results are shown for each patient. Patients II:2 and II:3 were immunized according to the recommended Belgian childhood immunization schedule that, among others, included tetanus, measles, mumps, rubella and 7-valent conjugated pneumococcal vaccines. Patient I:2 had received a tetanus booster vaccine within the last 10 years. A polysaccharide (unconjugated) pneumococcal vaccine was given to patients II:2 and I:2 at time of immunological evaluation; patient I:2 had never received a pneumococcal vaccine before then. Patients II:2 and II:3 were not vaccinated against varicella zoster virus but had chickenpox in early childhood. NA: not available. *Measured when not receiving immunoglobulin replacement therapy. †Reference values from Shearer *et al.*[27]. ‡Reference values from Piatosa *et al.*[28].

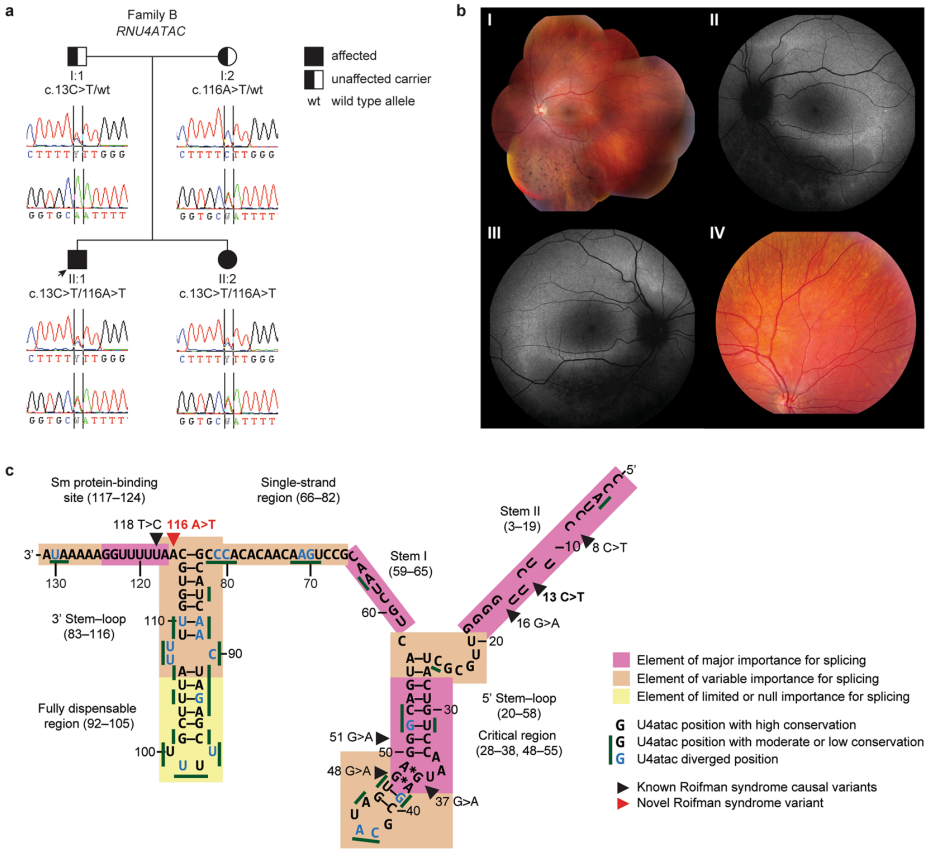


Figure 2. Family B with *RNU4ATAC*-associated Roifman syndrome (RS). (**a**) Pedigree of family B. (**b**) Representative retinal images of the RS patients. Panel I, composite retinal image of fundus of left eye (LE) of patient II:1: note inferior outer retinal atrophy with greyish hue and intraretinal pigment migration of the spicular type in inferior retina; mottled aspect of retinal pigment epithelium, more pronounced in inferotemporal area. Panel II, blue light autofluorescence image of LE of patient II:1 showing hyperautofluorescent delineation of inferior atrophic zone, as well as superior to optic disc, illustrating more widespread disease than can be seen on white light fundoscopic image only. Panel III, similar image of right eye (RE) of patient II:1 as in Panel II. Panel IV, fundus picture of detail of superonasal midperiphery of RE of patient II:2. Despite a normal full-field flash electroretinography, recent fundus examination at 14 years of age showed a mild mottling of pigment epithelium suggestive of early stage retinal dystrophy. (**c**) U4atac snRNA showing structural elements, conserved positions and location of variants associated with RS (adapted from ref. 8). The here-reported variant that has not been previously associated with RS is shown in red.

reduced switched memory B cells (Table 1). Taken together, the c.10835 + 1 G > A variant in *KMT2A* indicates a molecular diagnosis of WSS in the twin brothers and their mother.

Targeted sequencing confirms the diagnosis of RS in family B. In family B, WES was unable to identify a potentially disease-causing variant. In 2015, biallelic mutations in *RNU4ATAC* were identified in patients with RS[8]. Since *RNU4ATAC* is a noncoding snRNA gene, possible variants would have been missed with WES. Indeed, subsequent Sanger sequencing of *RNU4ATAC* revealed compound heterozygous variants in both siblings (c.13 C > T and c.116 A > T) that segregated in the parents (Fig. 2a). The c.13 C > T variant (rs559979281) had been previously reported in RS (Fig. 2c)[8]. The c.116 A > T variant has, to our knowledge, not yet been associated with human disease. The public database gnomAD (Genome Aggregation Database) contains two heterozygotes for the c.116 A > T variant (allele frequency of 0.00001531) but no homozygotes. Importantly, the c.116 A > T variant is located in a highly conserved position involved in splicing activity (Fig. 2c)[8]. Furthermore, position 116 is immediately adjacent to the Sm protein-binding site, which is a highly conserved structural element essential in splicing activity and previously implicated in RS (Fig. 2c)[8]. Together, the *RNU4ATAC* genotype confirms the diagnosis of RS in the family B siblings.

	Patient II:1		Patient II:2	
	Value	Reference range	Value	Reference range
White blood cells	*Age: 14 years*		*Age: 11 years*	
Total leukocyte count (no./μL)	7720	4500–12000	9800	4500–12000
Neutrophils (no./μL)	5290	2500–8000	6080	2500–8000
Lymphocytes (no./μL)	1190	1500–6500	2410	1500–6500
CD3+ T cells (no./μL)	940	800–3500	1740	800–3500
CD3 + CD4+ T helper cells (no./μL)	643	400–2100	1080	400–2100
CD45RA+ naive CD4+ T cells (%)	55	33–66†	68	33–66†
CD45RO+ memory CD4+ T cells (%)	38	18–38†	27	18–38†
CD3 + CD8+ T cytotoxic cells (no./μL)	274	200–1200	603	200–1200
CD45RA+ naive CD8+ T cells (%)	60	61–91†	67	61–91†
CD45RO+ memory CD8+ T cells (%)	33	4–23†	22	4–23†
CD19+ B cells (no./μL)	36	200–600	48	200–600
IgD + CD27- naive B cells (%)	77	51.3–82.5‡	70	51.3–82.5‡
CD24 + + CD38 + + transitional B cells (%)	15	1.4–13.0‡	5	1.4–13.0‡
IgD-CD27+ switched memory B cells (%)	9	8.7–25.6‡	10	8.7–25.6‡
IgD + CD27+ marginal zone B cells (%)	4	4.6–18.2‡	1	4.6–18.2‡
$CD21^{low}$ $CD38^{low}$ B cells (%)	22	2.7–8.7‡	21	2.7–8.7‡
CD3-CD56 + CD16+ NK cells (no./μL)	179	70–1200	554	70–1200
Monocytes (no./μL)	910	500–1000	960	500–1000
Eosinophils (no./μL)	290	100–500	230	100–500
Basophils (no./μL)	20	10–100	90	10–100
Immunoglobulins*	*Age: 3 years*		*Age: 6 years*	
IgG (g/L)	3.8	4.7–9.3	4.4	4.7–10.5
IgG2 (g/L)	0.53	0.63–3.0	0.49	0.85–4.1
IgG3 (g/L)	0.021	0.13–1.26	0.242	0.13–1.42
IgM (g/L)	<0.2	0.27–0.57	0.3	0.27–0.63
IgA (g/L)	0.3	0.41–0.91	<0.3	0.5–1.41
IgE (kU/L)	<4.4	0–60	<4.4	0–90
Isohemagglutinins*	*Age: 3 years*			
ABO blood type	O		NA	
Anti-A IgM	Negative	Positive	NA	
Anti-B IgM	Negative	Positive	NA	
Specific antibody responses*	*Age: 3 years*		*Age: 6 years*	
S. pneumoniae polysaccharide IgG (Lab U)	<3	≥11: immune	7	≥11: immune
Tetanus IgG (IU/mL)	0.03	≥0.01: immune	1	≥0.01: immune
Rubella IgG (IU/mL)	<8	>10: immune	NA	
Measles IgG (mIU/mL)	<150	>300: immune	NA	
Mumps IgG (Lab U/mL)	<230	>500: immune	NA	
Varicella zoster IgG (mIU/mL)	360	>100: immune	1400	>100: immune
Lymphocyte proliferation assay	*Age: 9 years*		*Age: 6 years*	
Response to Concanavalin A	Normal	Compared to control	Normal	Compared to control
Response to Phytohemagglutinin	Normal	Compared to control	Normal	Compared to control
Response to Tetanus toxoid	Normal	Compared to control	Normal	Compared to control

Table 2. Routine immunological laboratory results of the family B patients with Roifman syndrome. The most recent, comprehensive and representative laboratory results are shown for each patient. Both patients were immunized according to the recommended Belgian childhood immunization schedule that, among others, included tetanus, measles, mumps, rubella and 7-valent conjugated pneumococcal vaccines. A polysaccharide (unconjugated) pneumococcal vaccine was given at time of immunological evaluation. The patients were not vaccinated against varicella zoster virus but had chickenpox in early childhood. NA: not available. *Measured when not receiving immunoglobulin replacement therapy. †Reference values from Shearer *et al.*[27]. ‡Reference values from Piatosa *et al.*[28].

Immunological abnormalities in the WSS and RS patients. Because of the prominent immunodeficiency in both sibling pairs, we performed flow cytometric analysis of B and T lymphocyte subsets as previously described[19]. Interestingly, all patients from families A and B had decreased circulating follicular helper T (cTfh)

Clinical features†	18 published patients[7,9–14]	Present study		
		Patient II:2	Patient II:3	Patient I:2
Gender	8 M, 10 F	M	M	F
Age at last examination (years)	1–24	11	11	46
Short stature	18/18	+	+	+
Craniofacial features				
Microcephaly	2/7	−	−	−
Mild macrocephaly	NA	+	+	−
Hypertelorism, telecantus	9/17	+	+	+
Down-slanted palpebral fissures	14/16	+	+	+
Vertically narrow palpebral fissures	13/17	+	+	+
Strabismus	4/17	−	−	−
Thick eyebrows	14/17	+	+	+
Wide nasal bridge	16/18	+	+	+
Broad nasal tip	11/17	+	+	+
Long philtrum	2/12	−	−	−
Thin upper lip	6/12	+	+	+
Low-set ears	2/12	+	+	+
Abnormal dentition, hypodontia	5/9	−	−	−
High palate	4/8	+	+	+
Micrognathia	7/11	+	+	+
Musculoskeletal features				
Advanced bone age	1/16	NA	NA	NA
Small hands and feet	5/17	+	+	+
Fleshy hands and feet	3/7	+	+	+
Clinodactyly	8/18	−	+	+
Congenital hip dysplasia	2/17	−	−	−
Muscular hypotonia	9/18	−	−	−
Dermatological features				
Thick hair	14/17	+	+	+
Hypertrichosis cubiti	13/18	−	−	−
Hypertrichosis back and/or lower limbs	16/18	−	−	−
Neurological features				
Developmental or psychomotor delay	18/18	−	+	NA
Intellectual disability	16/17	+	+	+
Autism	2/12	−	−	−
Aggressive behavior	4/13	−	−	−
Hyperactivity	2/12	−	−	−
Seizures	1/7	−	−	−
Internal organ anomalies				
Cardiovascular anomalies	3/17	+	+	−
Urogenital anomalies	4/17	−	+	+
Intestinal anomalies	4/11	−	−	−
Feeding difficulties	10/18	+	+	−
Immunodeficiency features				
Antibody deficiency	1/1	+	+	+
Respiratory tract infections	2/17	+	+	+
Urinary tract infections	4/18	−	−	−
Bronchiectasis	NA	+	+	NA

Table 3. Comparison of the family A patients with published cases of *KMT2A*-associated Wiedemann-Steiner syndrome. NA: not available. †Not all clinical features have been ascertained in all previously published patients. Adapted from Stellacci *et al.*[7].

cells (Fig. 3a,b). Tfh cells play an essential role in the formation of antibody-producing plasma cells and memory B cells[20]. Furthermore, the two RS patients showed markedly reduced expression levels of B cell activating factor-receptor (BAFF-R), a receptor important in peripheral B cell survival (Fig. 3b)[21]. The WSS patients had normal BAFF-R levels (Fig. 3a). Expression of transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), a receptor related to BAFF-R, was normal in both the WSS and RS patients (Fig. 3a,b)[21].

Clinical features	6 published patients[8]	Present study	
		Patient II:1	Patient II:2
Gender	5 M, 1 F	M	F
Age at last examination (years)	NA	17	14
Growth retardation			
Prenatal, intra-uterine growth retardation	6/6	NA	NA
Postnatal growth retardation	6/6	+	+
Craniofacial features			
Mild microcephaly	5/6	+	+
Long philtrum	6/6	+	+
Thin upper lips	6/6	+	+
Narrow, tubular and upturned nose	6/6	+	+
Ophthalmological features			
Retinal dystrophy	3/6	+	+
Musculoskeletal features			
Epiphyseal dysplasia	6/6	+	−
Vertebral changes	3/6	+	−
Coxa vara	NA	+	−
Agenesis of anterior cruciate ligaments	NA	+	−
Agenesis of 12th ribs	NA	+	−
Short metacarpals	6/6	+	−
5th digit clinodactyly	4/6	−	−
Brachydactyly	6/6	+	−
Transverse palmar crease	5/6	−	−
Muscular hypotonia	5/6	−	−
Neurological features			
Intellectual disability, cognitive delay	5/6	−	−
Sensorineural hearing loss	1/6	−	−
Internal organ anomalies			
Noncompaction of the myocardium	1/6	−	−
Ventricular septum defect (VSD)	1/6	−	−
Lung hypoplasia	NA	−	+
Immunodeficiency and atopic features			
Antibody deficiency	6/6	+	+
Hepatosplenomegaly	5/6	−	−
Bronchiectasis	NA	+	+
Eczema	3/6	−	+

Table 4. Comparison of the family B patients with published cases of *RNU4ATAC*-associated Roifman syndrome. NA: not available.

For all patients, the alterations in naive and memory lymphocyte subsets (Supplementary Figs 1–4) corresponded with those seen in the routine laboratory assessment (Table 1). Other examined B and T cell populations fell within the range of the age-matched healthy controls (Supplementary Figs 1–4).

Discussion

We report two sibling pairs with an early-onset CVID phenotype as primary and cardinal presentation of WSS and RS: recurrent sinopulmonary infections, panhypogammaglobulinemia, reduced polysaccharide vaccine responses, and aberrant peripheral B cell subsets[1]. Because extra-immunological features were initially subtle and only became conspicuous with age, the establishment of an accurate diagnosis was significantly delayed. Therefore, we recommend to proactively evaluate all paediatric patients with a CVID phenotype for extra-immunological syndromic features, especially when presenting at an early age. In particular, diagnostic workup should include evaluation by a clinical geneticist, in addition to orthopedic, cardiologic, neurologic, urogenital and ophthalmologic assessment. To reach a conclusive diagnosis, genetic testing should be performed, varying from targeted testing of a specific disease gene to WES. Here, the diagnosis of WSS in family A was only confirmed upon WES in affected family members[7,9]. In family B on the other hand, WES failed to reveal the causal genetic defect because this was located in a noncoding gene[8]. Targeted testing of the known disease gene for RS allowed to identify the underlying mutations and to provide a definite diagnosis[8]. Of note, if WES does not identify a genetic defect and there is no known disease gene, whole genome sequencing should be undertaken[22].

With this study, we extend the phenotypical and mutational spectrum of both *KMT2A*-associated WSS and *RNU4ATAC*-associated RS[7–14]. In family A, we identified a novel heterozygous splice site mutation in *KMT2A*

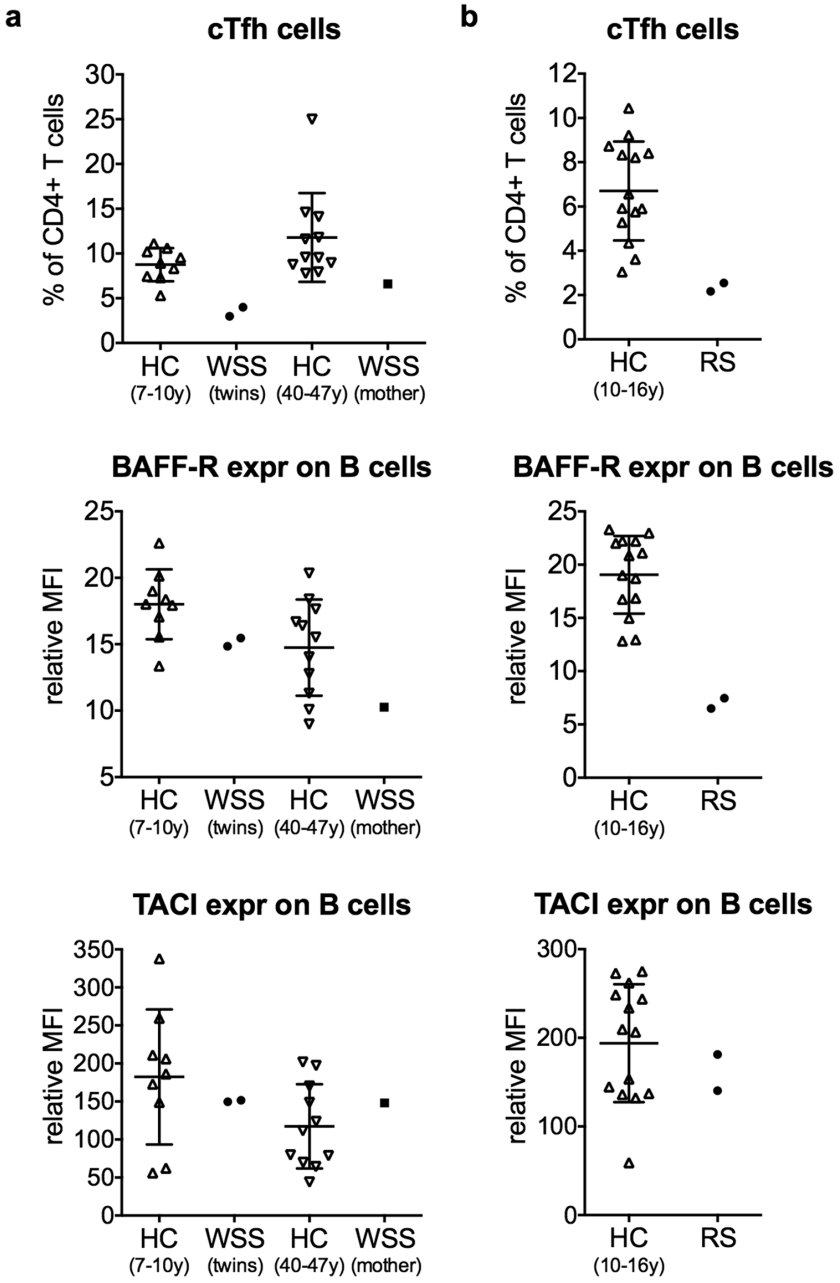


Figure 3. cTfh cells, BAFF-R expression and TACI expression in WSS and RS patients. (**a**) Family A patients with *KMT2A*-associated Wiedemann-Steiner syndrome (WSS). The twins (II:2, II:3) were 8 years old and the mother (I:2) was 43 years old at time of analysis. (**b**) Family B patients with *RNU4ATAC*-associated Roifman syndrome (RS). At time of analysis, the patients (II:1, II:2) were 14 and 11 years old, respectively. Flow cytometric immunophenotyping was performed on patients' PBMCs in comparison with age-matched healthy controls (HC). T cells were gated as $CD3^+$ and B cells as $CD19^+CD20^+$ in total PBMCs. Circulating follicular helper T (cTfh) cells were gated as $CXCR5^+CD45RO^+$ in $CD4^+$ T cells. BAFF-R and TACI expression were measured on B cells. Relative mean fluorescence intensity (MFI) was calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Graphs of the HC groups represent mean ± standard deviation. BAFF-R: B cell activating factor-receptor, cTfh: circulating follicular helper T, expr: expression, TACI: transmembrane activator and calcium modulator and cyclophilin ligand interactor.

causing in-frame deletion of exon 28. This deletion likely disrupts the stabilizing interaction site between the N- and C-terminal KMT2A fragments, resulting in loss of protein function[18]. So far, we were unable to confirm this on a protein level because the KMT2A-N protein fragment was not reliably detectable by western blot. Further studies on protein level are planned in the future. Family A is the first published kindred to show autosomal dominant transmission of *KMT2A*-associated WSS in multiple generations, as previously reported cases were either sporadic or parents were unavailable[7]. Remarkably, the characteristic hypertrichosis of elbows, back and/or lower limbs was absent in the here-reported WSS patients, confirming previous literature that this feature may not be as pathognomonic as initially believed[7,9,11,12]. Interestingly, *KMT2A*-associated WSS shows phenotypical overlap with Kabuki syndrome type 1 caused by heterozygous mutations in the related gene *KMT2D*[23]. Over 80% of patients with *KMT2D*-associated Kabuki syndrome develop defects in terminal B cell differentiation resulting in antibody deficiency[23]. Similarly, the here-described WSS patients demonstrated a block in terminal B cell differentiation evidenced by a relative increase in transitional and naive B cells and a relative decrease in switched memory B cells. Moreover, they had reduced levels of cTfh cells, which play a pivotal role in terminal B cell differentiation[20]. Decreased cTfh cells have, to our knowledge, not been previously reported in WSS or Kabuki syndrome. It would be interesting to investigate cTfh cells in additional patients with *KMT2A*-associated WSS and *KMT2D*-associated Kabuki syndrome as this may help elucidate the underlying pathophysiology of the antibody deficiency. In summary, humoral immune deficiency in patients with WSS reported by us and by Stellacci *et al.*[7], and antibody deficiency in patients with heterozygous mutations in the related *KMT2D* gene[23], strongly suggest a previously unknown role for *KMT2A* in B cell biology that may be related with T helper cell function.

In family B, we identified rare compound heterozygous mutations in the noncoding *RNU4ATAC* gene, of which one mutation (c.116 A > T) had not been associated with RS before[8]. Although it was not initially apparent, with time the boy showed typical features of RS including spondyloepiphyseal dysplasia and retinal dystrophy[8]. Curiously, he also demonstrated bilateral agenesis of the anterior cruciate ligaments and the 12th ribs, which are not typically seen in RS[8]. Spondyloepiphyseal or hip dysplasia have not yet been documented in the girl, currently 14 years old, although she displays mild growth retardation. Only recently, she was found to have mild fundus abnormalities suggestive of early stage retinal dystrophy. Note that retinal dystrophy was already evident in her brother at 7 years of age. It would be interesting to investigate why the girl has a milder phenotype than her brother. Since U4atac snRNA plays a role in minor intron splicing, it would be valuable to conduct RNA sequencing analysis in the two siblings and check for possible differences in intron retention[8]. Detailed immunological workup in the RS patients revealed markedly decreased BAFF-R expression on B cells. To our knowledge, this finding has not been previously published. As BAFF-R signalling is important for survival of B cells in the peripheral blood, this may provide an important clue towards the B cell lymphopenia seen in RS patients[21]. Moreover, analogous to the WSS patients, the RS siblings demonstrated decreased levels of cTfh cells, which may further compromise B cell differentiation and antibody production[20].

In conclusion, we here illustrate that a CVID phenotype can be the initial presentation of WSS and RS in early childhood while hallmark extra-immunological characteristics may be less prominent. With this, we highlight the importance of pursuing a genetic diagnosis in paediatric patients with an early-onset CVID phenotype, as this has important implications in terms of counselling, follow-up and screening for complications associated with the specific disorder.

Methods

Statement. All experiments and methods were carried out in accordance with relevant guidelines and regulations. The research protocol and all experimental protocols were approved by the ethical committee of Ghent University Hospital (2012/593). All reported subjects provided written informed consent for participation in the study, in accordance with the 1975 Helsinki Declaration.

Cytogenetic analyses. Microarray-based comparative genomic hybridization (array CGH) was performed on the affected sibling pairs of families A and B using the SurePrint G3 Human CGH Microarray Kit according to manufacturer's instructions (Agilent Technologies). Results were analyzed with arrayCGHbase[24]. Karyotype analysis was performed on the family A twins using the conventional G-banding technique. To screen the family A twins for submicroscopic subtelomeric rearrangements, multiplex ligation-dependent probe amplification (MLPA) analysis was performed using SALSA P070 and SALSA P036C probe mixes according to manufacturer's instructions (MRC-Holland). To examine the family A twins for 22q11.2 deletion, fluorescence *in situ* hybridization (FISH) analysis was performed using the DiGeorge Region Probe Set – LSI TUPLE 1 SpectrumOrange/LSI ARSA SpectrumGreen according to manufacturer's instructions (Vyvis).

WES. Genomic DNA was isolated from whole blood leukocytes using the Puregene DNA isolation kit (Qiagen) according to manufacturer's instructions. Whole exome enrichment was performed with the SureSelectXT Human All Exon V5 + UTRs kit (Agilent Technologies). Paired-end massively parallel sequencing (100 cycles) was performed on a NextSeq 500 (Illumina). Read mapping against the human genome reference sequence (NCBI, GRCh37), and post-mapping duplicate read removal, quality-based variant calling and coverage analysis were performed with CLC Genomics Workbench v6.0.4 (CLC bio). Sequencing coverage is summarized in Supplementary Table S1. Called variants with coverage ≥3 were annotated with Alamut Batch (Interactive Biosoftware). Only variants with population frequencies less than 10% were considered, according to public databases NCBI dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/), ExAC Browser (http://exac.broadinstitute.org/), and 1000 Genomes Project Browser (http://browser.1000genomes.org/). Variants were further prioritized based on allele frequency, functional prediction scores, nucleotide conservation scores and biological relevance[25]. Both Mendelian and non-Mendelian inheritance patterns were taken into account. Afterwards, variants of interest were evaluated using Alamut Visual

mutation interpretation software v2.7 rev. 1 (Interactive Biosoftware), Ingenuity Variant Analysis (QIAGEN, 2015 Release Spring), CADD scores v1.3 (http://cadd.gs.washington.edu/home), genome Aggregation Database (gnomAD) Browser (http://gnomad.broadinstitute.org), literature search, segregation analysis in available family members, and frequency in an in-house database containing variants of more than 1000 exomes at time of analysis.

Sanger sequencing of genomic DNA. DNA templates (GRCh37/hg19) of *KMT2A* (NM_001197104) and *RNU4ATAC* (NR_023343) were obtained from UCSC Genome Browser (https://genome.ucsc.edu). Primers for amplification and sequencing were designed with Primer3Plus[26]. For *KMT2A* exon 28 and adjacent intron-exon borders (family A): forward primer 5′-CAACCCACAAGGGTGTCTTC-3′ and reverse primer 5′-GCCCGGCTAATTCTTTTTGT-3′. For the unique exon and intron-exon borders of *RNU4ATAC* (family B): forward primer 5′-TGGAGGCTGGAGGTAAGCTA-3′ and reverse primer 5′-TGAGGTGCAAAGACCTACTGAA-3′. Genomic DNA was amplified by PCR using the specific primers and KAPA2G Robust Hotstart Ready Mix (KAPA Biosystems). PCR products were enzymatically purified with Exonuclease I and Antartic phosphatase (both New England BioLabs Inc.). Purified PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on a 3730xl DNA Analyzer (Applied Biosystems). Sequence reads were analyzed with SeqScape v2.5 (ThermoFisher Scientific).

RNA extraction, cDNA synthesis and confirmation of skipping of exon 28 in *KMT2A*. Total RNA was isolated from total PBMCs of all family A members and two control subjects by use of the RNeasy Plus Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad), according to manufacturer's instructions. The cDNA template (GRCh37/hg19) of *KMT2A* (NM_001197104) was obtained from UCSC Genome Browser (https://genome.ucsc.edu). Primers for amplification and sequencing of exon 28 and adjacent coding regions were designed with Primer3Plus[26]: forward primer 5′-AACCCAAACCAAAAACCAAAC-3′ and reverse primer 5′-CATCAGTGGGGAGCTGAAAT-3′. *GAPDH* was used as a reference target: forward primer 5′-CAGCCTCAAGATCATCAGCA-3′ and reverse primer 5′-TGTGGTCATGAGTCCTTCCA-3′. PCR amplification was performed by use of GoTaq Hot Start Colorless Master Mix (Promega). PCR products were analyzed on a 2% agarose gel in 1x TBE buffer (Quality Biological Inc). SYBR Safe (Invitrogen) signals were captured with a Gel Doc EZ Imager system (Bio-Rad). In addition, purified PCR products were Sanger sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on a 3130xL Genetic Analyzer (Applied Biosystems). Sequence reads were analyzed with SeqMan (DNAStar).

Flow cytometric analysis of PBMCs. Immunophenotyping was performed on PBMCs of patients and age-matched healthy controls. PBMCs were isolated from EDTA whole blood by Ficoll-Paque density gradient centrifugation and cryopreserved at −150 °C. Thawed PBMCs were stained with fixable viability dye 506 (eBioscience) and fluorescently labeled monoclonal antibodies under saturation conditions as previously described[19]. Cells were acquired on an LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo version X (Tree Star Inc.).

References

1. Bonilla, F. A. *et al.* International Consensus Document (ICON): Common Variable Immunodeficiency Disorders. *J. Allergy Clin. Immunol. Pract* **4**, 38–59, doi:10.1016/j.jaip.2015.07.025 (2016).
2. Bogaert, D. J. *et al.* Genes associated with common variable immunodeficiency: one diagnosis to rule them all? *J. Med. Genet.* **53**, 575–590, doi:10.1136/jmedgenet-2015-103690 (2016).
3. Resnick, E. S., Moshier, E. L., Godbold, J. H. & Cunningham-Rundles, C. Morbidity and mortality in common variable immune deficiency over 4 decades. *Blood* **119**, 1650–1657, doi:10.1182/blood-2011-09-377945 (2012).
4. Ming, J. E., Stiehm, E. R. & Graham, J. M. Jr. Syndromic immunodeficiencies: genetic syndromes associated with immune abnormalities. *Crit. Rev. Clin. Lab. Sci.* **40**, 587–642, doi:10.1080/714037692 (2003).
5. Kersseboom, R., Brooks, A. & Weemaes, C. Educational paper: syndromic forms of primary immunodeficiency. *Eur. J. Pediatr.* **170**, 295–308, doi:10.1007/s00431-011-1396-7 (2011).
6. Ming, J. E. & Stiehm, E. R. Genetic syndromic immunodeficiencies with antibody defects. *Immunol. Allergy Clin. North. Am.* **28**, 715–736, vii, doi:10.1016/j.iac.2008.06.007 (2008).
7. Stellacci, E. *et al.* Congenital immunodeficiency in an individual with Wiedemann-Steiner syndrome due to a novel missense mutation in KMT2A. *Am. J. Med. Genet. A* **170**, 2389–2393, doi:10.1002/ajmg.a.37681 (2016).
8. Merico, D. *et al.* Compound heterozygous mutations in the noncoding RNU4ATAC cause Roifman Syndrome by disrupting minor intron splicing. *Nat. Commun.* **6**, 8718, doi:10.1038/ncomms9718 (2015).
9. Jones, W. D. *et al.* De novo mutations in MLL cause Wiedemann-Steiner syndrome. *Am. J. Hum. Genet.* **91**, 358–364, doi:10.1016/j.ajhg.2012.06.008 (2012).
10. Mendelsohn, B. A., Pronold, M., Long, R., Smaoui, N. & Slavotinek, A. M. Advanced bone age in a girl with Wiedemann-Steiner syndrome and an exonic deletion in KMT2A (MLL). *Am. J. Hum. Genet. A* **164a**, 2079–2083, doi:10.1002/ajmg.a.36590 (2014).
11. Strom, S. P. *et al.* De Novo variants in the KMT2A (MLL) gene causing atypical Wiedemann-Steiner syndrome in two unrelated individuals identified by clinical exome sequencing. *BMC Med. Genet.* **15**, 49, doi:10.1186/1471-2350-15-49 (2014).
12. Calvel, P. *et al.* A Case of Wiedemann-Steiner Syndrome Associated with a 46, XY Disorder of Sexual Development and Gonadal Dysgenesis. *Sex. Dev.* **9**, 289–295, doi:10.1159/000441512 (2015).
13. Dunkerton, S. *et al.* A de novo Mutation in KMT2A (MLL) in monozygotic twins with Wiedemann-Steiner syndrome. *Am. J. Med. Genet. A* **167a**, 2182–2187, doi:10.1002/ajmg.a.37130 (2015).
14. Miyake, N. *et al.* Delineation of clinical features in Wiedemann-Steiner syndrome caused by KMT2A mutations. *Clin. Genet.* **89**, 115–119, doi:10.1111/cge.12586 (2016).
15. Yokoyama, A., Kitabayashi, I., Ayton, P. M., Cleary, M. L. & Ohki, M. Leukemia proto-oncoprotein MLL is proteolytically processed into 2 fragments with opposite transcriptional properties. *Blood* **100**, 3710–3718, doi:10.1182/blood-2002-04-1015 (2002).
16. Roifman, C. M. Antibody deficiency, growth retardation, spondyloepiphyseal dysplasia and retinal dystrophy: a novel syndrome. *Clin. Genet.* **55**, 103–109, doi:10.1034/j.1399-0004.1999.550206.x (1999).
17. Gray, P. E., Sillence, D. & Kakakios, A. Is Roifman syndrome an X-linked ciliopathy with humoral immunodeficiency? Evidence from 2 new cases. *Int. J. Immunogenet.* **38**, 501–505, doi:10.1111/j.1744-313X.2011.01041.x (2011).

18. Hsieh, J. J., Ernst, P., Erdjument-Bromage, H., Tempst, P. & Korsmeyer, S. J. Proteolytic cleavage of MLL generates a complex of N- and C-terminal fragments that confers protein stability and subnuclear localization. *Mol. Cell. Biol.* **23**, 186–194, doi:10.1128/MCB.23.1.186-194.2003 (2003).
19. Bogaert, D. J. *et al.* The immunophenotypical fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. *Haematologica* **102**, 192–202, doi:10.3324/haematol.2016.149112 (2017).
20. Liu, X., Nurieva, R. I. & Dong, C. Transcriptional regulation of follicular T-helper (Tfh) cells. *Immunol. Rev.* **252**, 139–145, doi:10.1111/imr.12040 (2013).
21. Rickert, R. C., Jellusova, J. & Miletic, A. V. Signaling by the tumor necrosis factor receptor superfamily in B-cell biology and disease. *Immun. Rev* **244**, 115–133, doi:10.1111/j.1600-065X.2011.01067.x (2011).
22. Meienberg, J., Bruggmann, R., Oexle, K. & Matyas, G. Clinical sequencing: is WGS the better WES? *Hum. Genet.* **135**, 359–362, doi:10.1007/s00439-015-1631-9 (2016).
23. Lindsley, A. W. *et al.* Defects of B-cell terminal differentiation in patients with type-1 Kabuki syndrome. *J. Allergy Clin. Immunol.* **137**, 179–187.e110, doi:10.1016/j.jaci.2015.06.002 (2016).
24. Menten, B. *et al.* arrayCGHbase: an analysis platform for comparative genomic hybridization microarrays. *BMC bioinformatics* **6**, 124, doi:10.1186/1471-2105-6-124 (2005).
25. Coppieters, F. *et al.* Identity-by-descent-guided mutation analysis and exome sequencing in consanguineous families reveals unusual clinical and molecular findings in retinal dystrophy. *Genet. Med.* **16**, 671–680, doi:10.1038/gim.2014.24 (2014).
26. Untergasser, A. *et al.* Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res* **35**, W71–74, doi:10.1093/nar/gkm306 (2007).
27. Shearer, W. T. *et al.* Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. *J. Allergy Clin. Immunol.* **112**, 973–980, doi:10.1016/j.jaci.2003.07.003 (2003).
28. Piatosa, B. *et al.* B cell subsets in healthy children: reference values for evaluation of B cell maturation process in peripheral blood. *Cytometry B Clin. Cytom* **78**, 372–381, doi:10.1002/cyto.b.20536 (2010).

Acknowledgements

The authors gratefully acknowledge the families who participated in this study. This study was supported by the Ghent University Hospital Spearhead Initiative for Immunology Research, the Jeffrey Modell Foundation to F.H., the Research Foundation Flanders (FWO) to D.B., B.P.L, F.C., B.N.L. and E.D.B., the Ghent University Special Research Fund (BOF15/GOA/011) to E.D.B., Hercules foundation AUGE/13/023 to E.D.B., a European Research Council consolidator grant to B.N.L., an Interuniversity Attraction Pole grant to B.N.L., the University of Ghent MRP program "Group-ID" to B.N.L., and the NIH Clinical Center intramural research program to H.S.K., J.E.N. and S.D.R. D.B. is a PhD fellow, F.C. a postdoctoral fellow, and E.D.B. and B.P.L. are Senior Clinical Investigators of the FWO.

Author Contributions

D.J.B. performed the genetic analyses, the experiments and data analysis, and drafted the initial manuscript. M.D., H.S.K. and S.D.R. supervised experiments and data analysis, and critically reviewed and revised the manuscript. J.E.N. assisted in protein structure analysis and critically reviewed and revised the manuscript. B.P.L., H.D.W., S.D.S., B.N.L. and F.D.B. managed patients, provided clinical data and critically reviewed and revised the manuscript. M.D.B. and F.C. assisted in genetic analyses and critically reviewed and revised the manuscript. E.D.B. supervised genetic analyses and critically reviewed and revised the manuscript. F.H. conceptualized the study, managed patients, provided and interpreted clinical data, and critically reviewed and revised the manuscript. All authors provided critical input and approved the final manuscript as submitted.

Additional Information

Supplementary information accompanies this paper at doi:10.1038/s41598-017-02434-4

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information

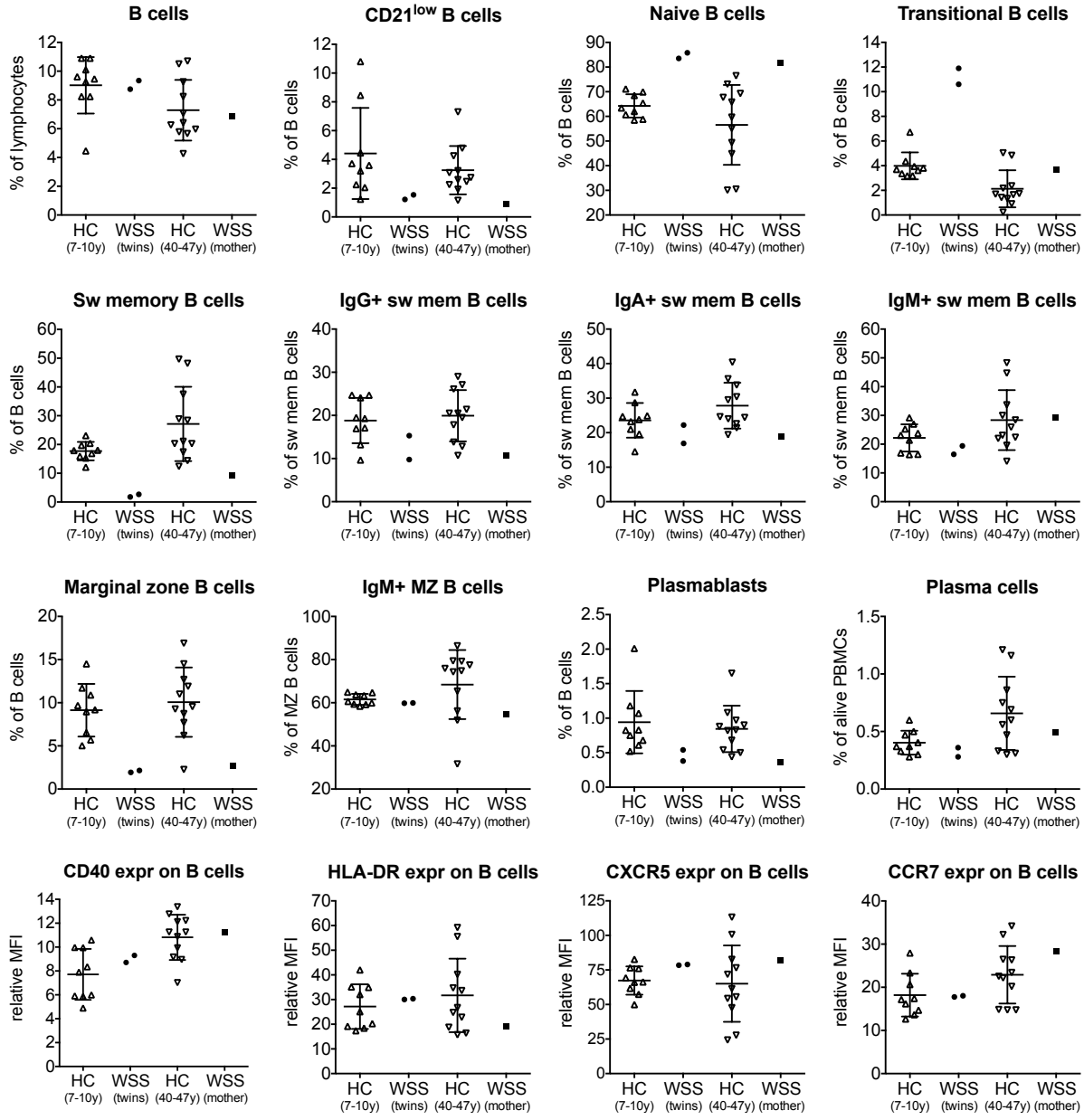
SUPPLEMENTARY TABLE

Supplementary Table 1. WES coverage.

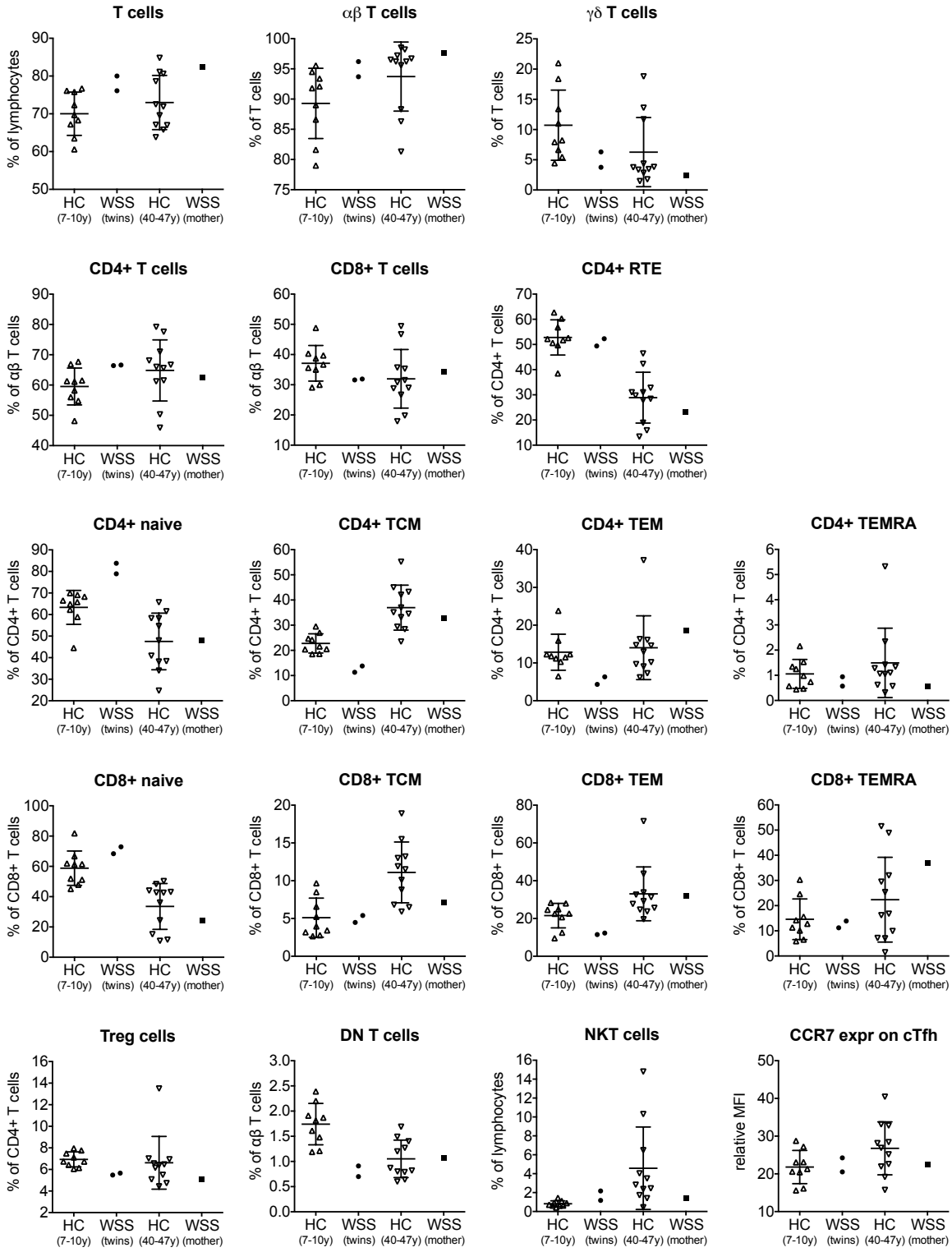
Family	Subject	Read depth				
		Average read depth	% of regions ≥ 5 x	% of regions ≥ 10 x	% of regions ≥ 20 x	% of regions ≥ 40 x
A	I:1	74.8 x	98.79%	97.75%	93.52%	75.81%
A	I:2	67.9 x	98.59%	97.39%	92.48%	71.62%
A	II:3	67.6 x	98.69%	97.47%	92.42%	71.62%
B	I:1	61.5 x	96.12%	93.80%	86.31%	63.60%
B	I:2	73.7 x	95.82%	93.82%	88.28%	71.08%
B	II:1	76.7 x	98.90%	97.85%	93.28%	75.34%
B	II:2	65.9 x	95.94%	93.75%	87.09%	67.07%

Read depth was calculated for all regions enriched by the SureSelectXT Human All Exon V5+UTRs kit (Agilent Technologies, CA, USA), using CLC Genomics Workbench v6.0.4 (CLC bio, MA, USA).

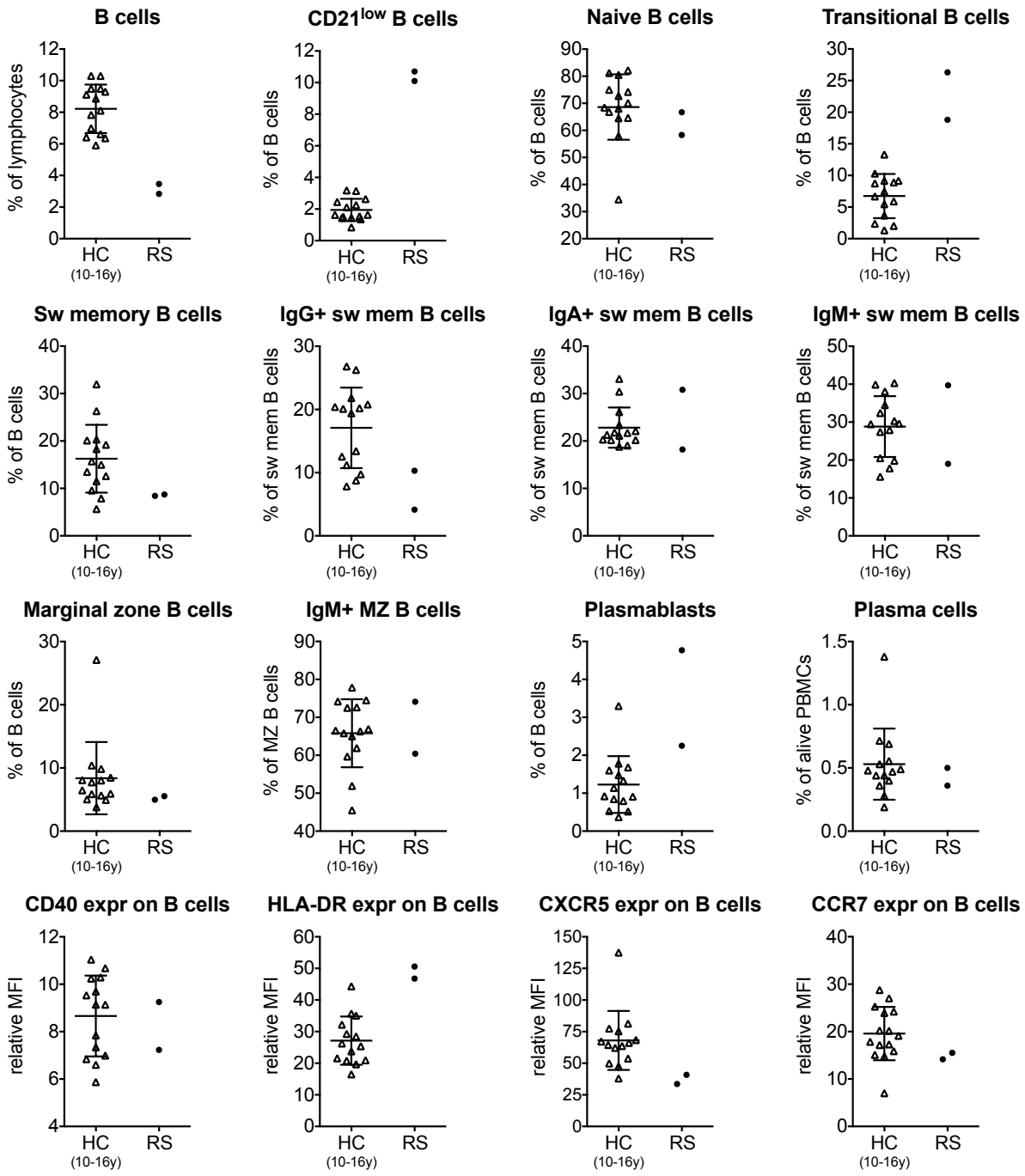
SUPPLEMENTARY FIGURES



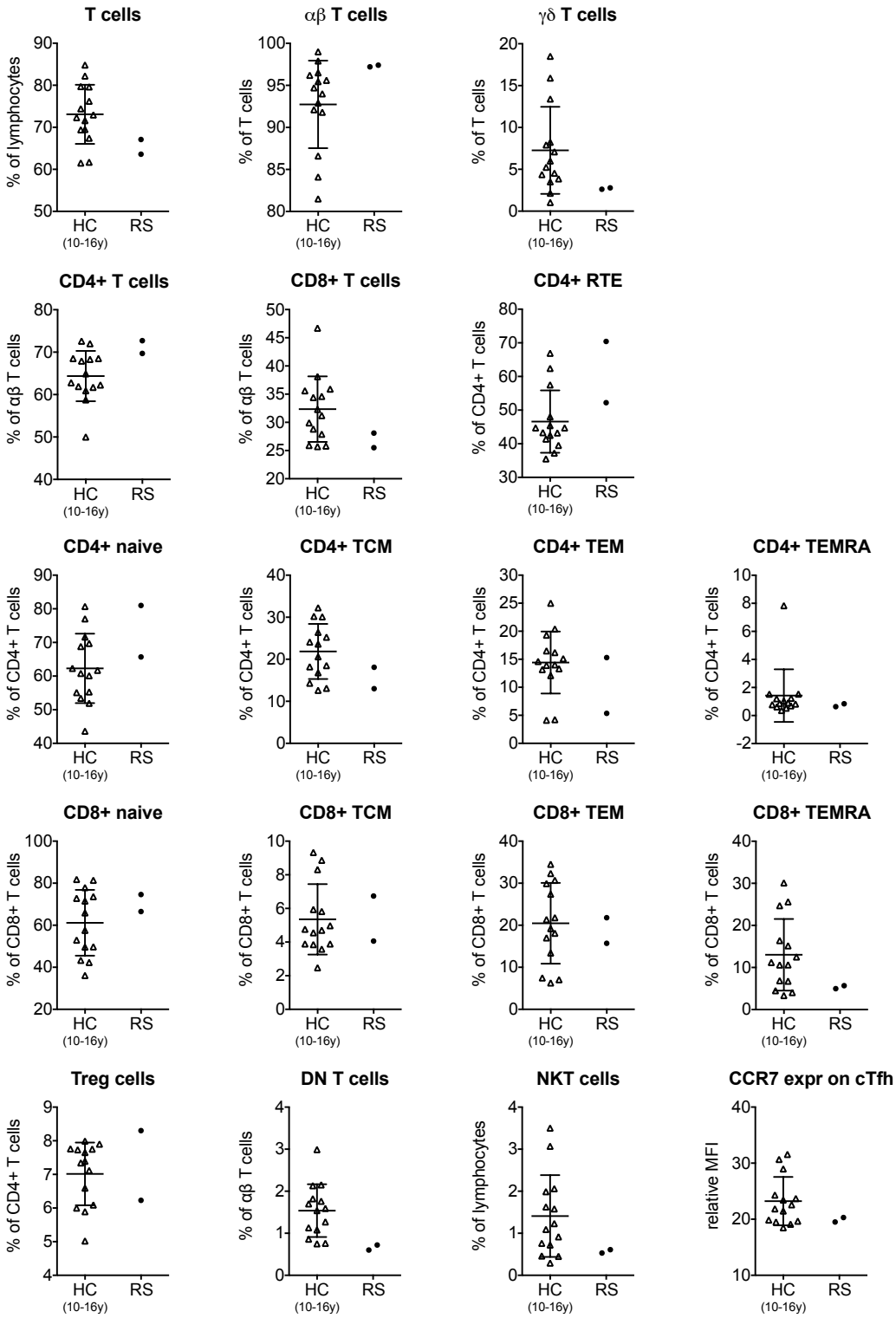
Supplementary Figure 1. Flow cytometric phenotyping of the family A patients: B cell subsets. Flow cytometric immunophenotyping was performed on PBMCs of the 3 patients with *KMT2A*-associated Wiedemann-Steiner syndrome (WSS) in comparison with age-matched healthy controls (HC). At time of analysis, the twins and the mother were 8 and 43 years old, respectively. For details on gating, see reference 19. Relative mean fluorescence intensity (rMFI) was calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Graphs of HC group represent mean ± standard deviation. MZ: marginal zone, sw (mem): switched (memory); expr, expression.



Supplementary Figure 2. Flow cytometric phenotyping of the family A patients: T cell subsets. Flow cytometric immunophenotyping was performed on PBMCs of the 3 patients with KMT2A-associated Wiedemann-Steiner syndrome (WSS) in comparison with age-matched healthy controls (HC). At time of analysis, the twins and the mother were 8 and 43 years old, respectively. For details on gating, see reference 19. Relative mean fluorescence intensity (rMFI) was calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Graphs of HC group represent mean ± standard deviation. cTfh: circulating follicular helper T, DN: double negative, expr: expression, NKT: natural killer T, RTE: recent thymic emigrants, TCM: central memory T, TEM: effector memory T, TEMRA: effector memory RA T, Treg: regulatory T.



Supplementary Figure 3. Flow cytometric phenotyping of the family B patients: B cell subsets. Flow cytometric immunophenotyping was performed on PBMCs of the 2 patients with *RNU4ATAC*-associated Roifman syndrome (RS) in comparison with age-matched healthy controls (HC). At time of analysis, the patients (II:1, II:2) were 14 and 11 years old, respectively. For details on gating, see reference 19. Relative mean fluorescence intensity (rMFI) was calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Graphs of HC group represent mean ± standard deviation. MZ: marginal zone, sw (mem): switched (memory), expr: expression.



Supplementary Figure 4. Flow cytometric phenotyping of the family B patients: T cell subsets. Flow cytometric immunophenotyping was performed on PBMCs of the 2 patients with RNU4ATAC-associated Roifman syndrome (RS) in comparison with age-matched healthy controls (HC). At time of analysis, the patients (II:1, II:2) were 14 and 11 years old, respectively. For details on gating, see reference 19. Relative mean fluorescence intensity (rMFI) was calculated by dividing the MFI of the positive population by the MFI of the Fluorescence Minus One (FMO) population. Graphs of HC group represent mean ± standard deviation. cTfh: circulating follicular helper T, DN: double negative, expr: expression, NKT: natural killer T, RTE: recent thymic emigrants, TCM: central memory T, TEM: effector memory T, TEMRA: effector memory RA T, Treg: regulatory T.

Chapter 6

A novel IKAROS haploinsufficiency kindred with unexpectedly late and variable B cell maturation defects

6 A NOVEL *IKAROS* HAPLOINSUFFICIENCY KINDRED WITH UNEXPECTEDLY LATE AND VARIABLE B CELL MATURATION DEFECTS

Delfien J. Bogaert, MD[1,2,3,4,5], Hye Sun Kuehn, PhD[6], Carolien Bonroy, MPharm, PhD[4,7], Katherine R. Calvo, MD, PhD[8], Joke Dehoorne, MD, PhD[9], Arnaud V. Vanlander, MD, PhD[10], Marieke De Bruyne, MSc[1,2,3,4], Urszula Cytlak, PhD[11,12], Venetia Bigley, MD, PhD[11,12], Frans De Baets, MD, PhD[2,4], Elfride De Baere, MD, PhD[3,4], Sergio D. Rosenzweig, MD, PhD[6], Filomeen Haerynck, MD, PhD[1,2,4,*], Melissa Dullaers, PhD[1,4,5,13,*]

[1]Clinical Immunology Research Lab, Department of Pulmonary Medicine, Ghent University Hospital, Ghent, Belgium; [2]Department of Pediatrics, Division of Pediatric Immunology and Pulmonology, Ghent University Hospital, Ghent, Belgium; [3]Center for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium; [4]Center for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, Belgium; [5]Laboratory of Immunoregulation, VIB Inflammation Research Center, Ghent, Belgium; [6]Immunology Service, Department of Laboratory Medicine, NIH Clinical Center, National Institutes of Health, Bethesda, MD, USA; [7]Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium; [8]Hematology Section, Department of Laboratory Medicine, NIH Clinical Center, National Institutes of Health, Bethesda, MD, USA; [9]Department of Pediatrics, Division of Pediatric Nephrology and Rheumatology, Ghent University Hospital, Ghent, Belgium; [10]Department of Pediatrics, Division of Pediatric Neurology and Metabolism, Ghent University Hospital, Ghent, Belgium; [11]Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom; [12]Northern Centre for Bone Marrow Transplantation, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, United Kingdom; [13]Department of Internal Medicine, Ghent University, Ghent, Belgium; *FH and MD contributed equally to this work.

Accepted for publication by The Journal of Allergy and Clinical Immunology.

CAPSULE SUMMARY

We report on the first truncating *IKZF1* mutation associated with IKAROS haploinsufficiency and illustrate an unexpectedly late and variable block in central and peripheral B cell development in two patients and their asymptomatic mother.

To the Editor:

IKAROS, encoded by *IKAROS family zinc finger protein 1* (*IKZF1*), is a hematopoietic zinc finger transcription factor essential in human hematopoiesis and B cell development.[1] Somatic changes in *IKZF1* have been associated with B cell leukemia.[1] Germline *IKZF1* mutations have been recently identified as a cause of common variable immunodeficiency and dysgammaglobulinemia through IKAROS haploinsufficiency.[2,3] They are autosomal dominantly inherited or appear *de novo*.[2-4] Eleven out of 42 germline *IKZF1* mutation carriers were clinically asymptomatic at time of publication, suggesting incomplete penetrance.[2-4] However, as age of onset ranged from infancy to the sixth decade, younger asymptomatic subjects may still develop disease later in life.[2,3] Symptomatic individuals mainly presented bacterial infections, especially of the respiratory tract.[2,3] In addition, some patients suffered from antibody-mediated autoimmune manifestations or B cell acute lymphoblastic leukemia.[2,3] Affected subjects typically showed a (progressive) decrease of peripheral B cell numbers accompanied by a (progressive) reduction in at least one major immunoglobulin (Ig) isotype.[2,3] Total T cells were not decreased but patients often displayed quantitative changes in various T cell subsets.[2,3] Here, we report an autosomal dominant kindred with a novel truncating mutation in *IKZF1*, and describe new insights in B cell maturation in two symptomatic and one asymptomatic family members.

The parents (I:1, I:2) were healthy, non-consanguineous, and from Dutch-Italian origin (Figure 1A). Sibling II:3, currently 17 years old, was diagnosed with monocyclic systemic-onset juvenile idiopathic arthritis at the age of six, and reactive arthritis at nine years of age. Testing for autoantibodies was negative. Her younger sister (II:4, currently 12.5 years old) had multiple episodes of reactive arthritis between the ages of three and five. In addition, both patients suffered from recurrent bacterial sinopulmonary infections. Patient II:4 was recently diagnosed with juvenile myasthenia gravis confirmed by positive acetylcholine receptor auto-antibodies. The immunological workup of patients II:3 and II:4 is provided in Table E1 and Figure E1 in this article's Online Repository. In summary, patient II:3, first examined at the age of nine years, had absent serum IgA and IgM and severe B lymphopenia (Figure 2A). Patient II:4, first evaluated at 4.5 years of age, showed absent IgA but normal IgM. Her B cell counts were initially in the lower normal range but progressively decreased over time (Figure 2A). Both patients had poor polysaccharide vaccination responses. Total IgG and IgG subclass levels have so far remained normal. There were no important abnormalities in peripheral T cell subsets. The youngest sibling (II:5) is currently 8 years old and in good health.

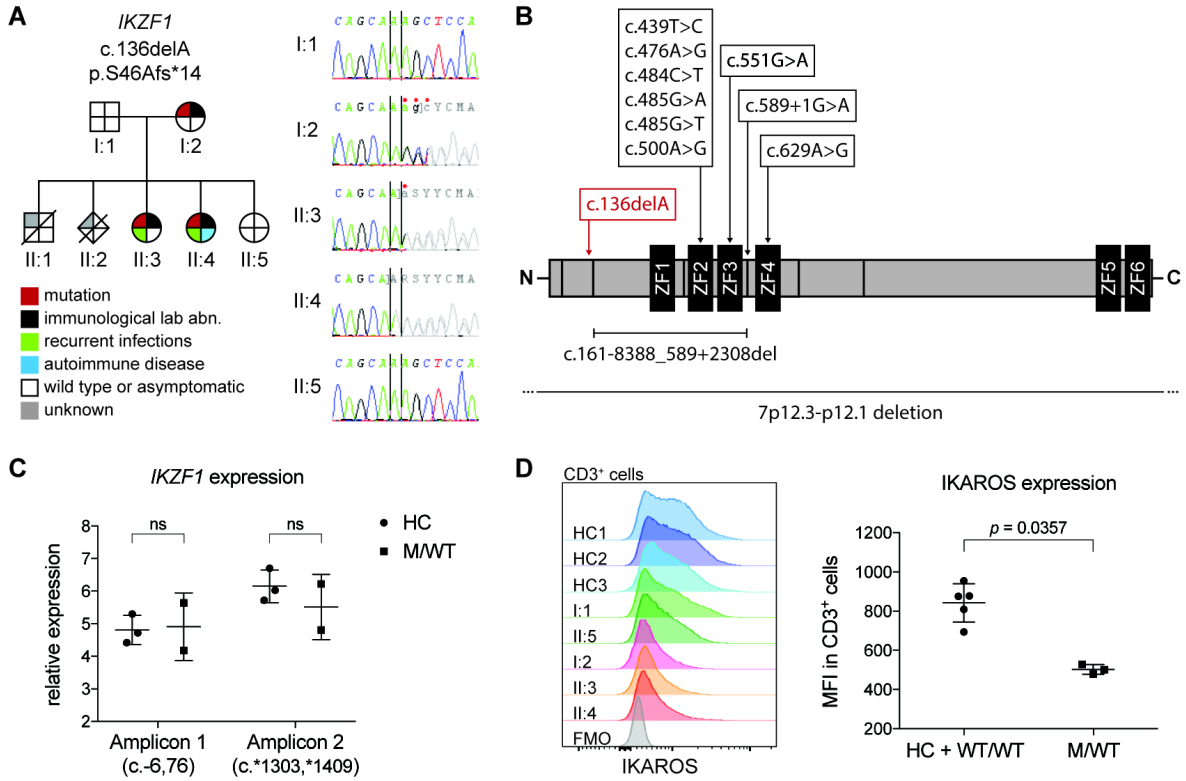


Figure 1. Molecular and functional characterization. (A) Pedigree. Square, circle and diamond shapes indicate male, female and sex unknown, respectively. Diagonal lines indicate deceased siblings. Corresponding electropherograms are shown on the right. Lab abn.: laboratory abnormalities. **(B)** Schematic structure of IKAROS isoform 1 (*IKZF1* transcript NM_006060). Grey boxes represent exons. Zinc fingers (ZF) 1 to 4 constitute the DNA binding domain, ZF5 and ZF6 the protein dimerization domain. Previously published mutations are depicted in black; one family had a large chromosomal deletion (7p12.3-p12.1) encompassing the *IKZF1* gene. The here-reported novel mutation is indicated in red. **(C)** *IKZF1* mRNA expression was determined with real-time quantitative PCR by means of two amplicons located upstream and downstream of the mutation site, respectively. The cDNA start and end position of each amplicon is given between brackets (*IKZF1* transcript NM_006060). The two studied patients (II:3, II:4) had similar *IKZF1* mRNA levels compared to healthy controls (HC) indicating that the mutant transcript escapes nonsense-mediated mRNA decay. The graph represents mean ± SD of one experiment. ns: not significant. **(D)** IKAROS protein expression in mutant (M/WT) and wild type (WT/WT) family members and three healthy controls (HC) analyzed in $CD3^+$ T cells by use of flow cytometry. Histograms are depicted on the left. The graph on the right displays mean ± SD of the corresponding mean fluorescence intensity (MFI) values. Data shown are representative of two replicate experiments. FMO: fluorescence minus one.

Whole exome sequencing revealed a heterozygous frameshift mutation in *IKZF1* (NM_006060: c.136delA, p.S46Afs*14) in the two affected siblings (II:3, II:4) and asymptomatic mother (I:2) (Figure 1A). The mutation is not reported in public or in-house databases. The frameshift introduces a premature stopcodon before the first zinc finger motif in all *IKZF1* transcripts (Figure 1B). Although nonsense-mediated mRNA decay (NMD) was predicted *in silico*, mutant transcripts were detected at cDNA level (Figure E2 in this article's Online Repository) and patients showed normal levels of *IKZF1* cDNA (Figure 1C),

suggesting mutant transcripts escape NMD. Nonetheless, IKAROS protein expression levels were, on average, 40% lower in all three mutant subjects compared to the wild type family members and healthy controls (Figure 1D). The latter suggests that the mutant transcript encodes an unstable truncated protein that is rapidly degraded.[5] Alternatively, the truncated protein may not be recognized by the antibody, however, epitope details are lacking. Either way, our findings indicate a molecular diagnosis of IKAROS haploinsufficiency in patients II:3 and II:4 and their asymptomatic mother (I:2). Because the mutation was also present in the clinically asymptomatic 43-year-old subject I:2, we performed in-depth immunological testing, which revealed mildly reduced serum IgM as well as marginally inadequate antibody responses to a polysaccharide pneumococcal vaccine (Table E1 in this article's Online Repository).

Since germline IKAROS haploinsufficiency is a newly identified primary immunodeficiency (PID) and data on asymptomatic mutation carriers is scarce, we evaluated the peripheral and central B cell compartment both in the affected siblings and in their asymptomatic mother. In patient II:3, the few circulating B cells consisted for 50% of transitional B cells (Figure 2B). Total naive B cell levels, encompassing transitional B cells, were also increased at the expense of switched memory and marginal zone B cells (Figure 2B). In patient II:4, normal numbers of peripheral B cell subsets revealed an arrest at a later stage in development. Particularly, transitional B cell levels were normal, whereas total naive B cells were relatively increased and memory-type B cell percentages were decreased (Figure 2B). This points towards a block after the naive mature B cell stage. Analogous to previously reported asymptomatic *IKZF1* mutation carriers[2,3], the asymptomatic mother (I:2) exhibited peripheral B cell frequencies and subsets within normal ranges for age, although memory-type B cells were at the lower end and naive B cells at the upper end. This remained stable over a period of about two years (Figure 2B).

Bone marrow aspirates from patient II:3 and asymptomatic mother (I:2) revealed normal frequencies of hematopoietic stem cells (HSC). Multipotent progenitors (MPP) were reduced, while common lymphoid progenitors (CLP) were moderately expanded (Figure 2C and Figure E3 in this article's Online Repository) and total B-lineage cells were profoundly decreased in both patients (Figure 2C and Figure 2D, upper panel). Symptomatic patient II:3 exhibited additionally a partial block from pro- to pre-B cells (Figure 2D, middle panel) and an almost complete block from immature/transitional to mature B cells (Figure 2D, lower panel). In the asymptomatic mother I:2 on the other hand, the reduced B-lineage cells demonstrated a normal maturation profile and were sufficient to sustain normal peripheral B cell numbers. Patient II:4 was unavailable for bone marrow analysis.

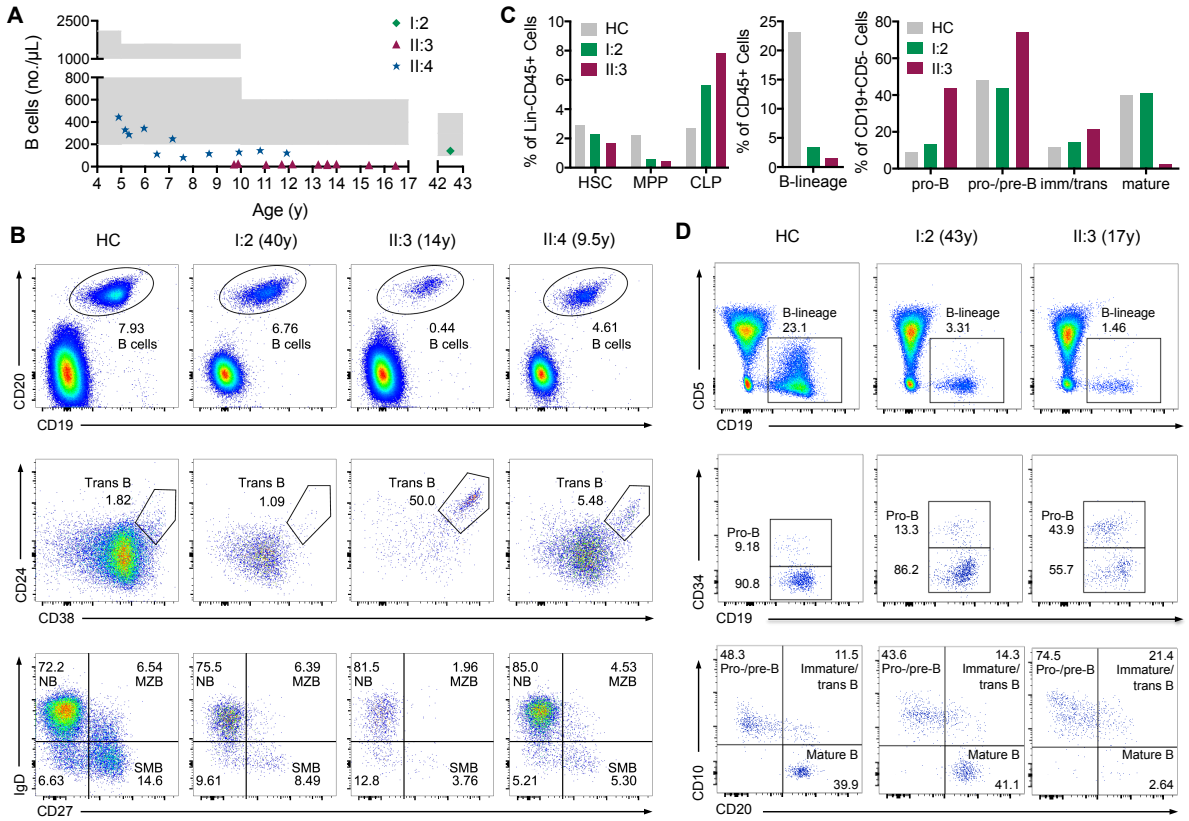


Figure 2. B cell phenotype. (A) Peripheral blood total B cell counts. Measurements shown are from the first to the last immunological laboratory evaluation performed in our hospital. Grey shading represents the age-based reference range. **(B)** Peripheral blood B cell subsets. The top row depicts total B cells gated as $CD19^+CD20^+$ in alive peripheral blood mononuclear cells. In the second and third rows, B cell subsets were gated on total $CD19^+CD20^+$ B cells as indicated. Note that the samples shown here were taken at different time points than those in Table E1 of this article's Online Repository. **(C)** Overview of different precursor stages of B cell development in bone marrow, shown as proportion of total Lin^-CD45^+, $CD45^+$ or $CD19^+CD5^-$ cells as indicated. **(D)** Bone marrow B cell subsets. The top row depicts total B-lineage cells gated as $CD19^+CD5^-$ in $CD45^+$ cells. In the second and third rows, B-lineage subsets were gated within this $CD19^+CD5^-$ population. The middle graphs allow discrimination of pro-B cells ($CD34^+CD19^+$) from more mature B-lineage cells ($CD34^-CD19^+$). The lower graphs show the development from pro- and pre-B cells (pro-/pre-B; $CD10^+CD20^-$) over immature and transitional B cells (immature/trans B; $CD10^+CD20^+$) to mature naive B cells (mature B, $CD10^-CD20^+$). Abbreviations: No.: number, y: years, HC: healthy control, MZB: marginal zone B cells, NB: naive B cells, SMB: switched memory B cells, Trans B: transitional B cells, HSC: hematopoietic stem cells, MPP: multipotent progenitors, CLP: common lymphoid progenitors, imm: immature B.

The multi-level central B cell developmental block observed in this *IKZF1* family appears later than observed in previously published IKAROS haploinsufficient cases.[2,3] First, early hematopoiesis in I:2 and II:3 showed a partial arrest at the MPP and the B-lineage stages, whereas two patients reported by Hoshino et al. exhibited blockages at the earlier HSC and CLP stages in addition to the B-lineage block.[3] Second, within B-lineage cells, symptomatic patient II:3 exhibited arrests at the pre-B and mature B cell stages, whereas the Hoshino patients showed normal composition of the B-lineage and two patients described by Kuehn et al. had a block at the earlier pro-B cell stage[2]. Whether these differences are linked to the

genotype of our patients remains to be established, since all four previously reported patients harbored missense mutations in the second zinc finger motif of *IKZF1*, whereas our patients have a loss of IKAROS protein due to a frameshift mutation.

To our knowledge, there have been no previous reports on central B cell development in asymptomatic *IKZF1* mutation carriers. In subject I:2, the absence of clinical involvement was reflected in a quasi-normal peripheral B cell development. However, considering the important reduction in central B-lineage cells and the recently documented specific antibody deficiency, the B cell compartment is clearly affected and she might still become symptomatic at a later age.[2] Interestingly, a Kaplan-Meier curve for onset of symptoms generated based on 45 reported symptomatic and asymptomatic cases, suggests that all germline *IKZF1* mutation carriers may eventually go on to develop symptoms (see Figure E4 in this article's Online Repository). Although Kaplan-Meier estimates at the utmost ends of a time interval need to be interpreted with caution, from a clinical perspective this warrants longitudinal follow-up of asymptomatic subjects. Peripheral B cell numbers seem to be a suitable marker for monitoring disease progression, whereas serum IgG levels have been shown to remain normal for years following the development of B cell lymphopenia.[2]

IKAROS haploinsufficiency shares important genetic aspects with other recently identified PIDs of haploinsufficiency. Notable are NF-κB1, CTLA-4 and GATA2 haploinsufficiencies, disorders that also affect the B cell compartment and are characterized by incomplete penetrance as well as variable expressivity and a highly variable age of onset in manifesting individuals.[6,7] The molecular basis of incomplete penetrance and variable expressivity is poorly understood.[6] Murine models have implicated an important role for IKAROS at nearly every step of B cell development and function.[8,9] Besides the more obvious defects in hematopoietic progenitor cell development, impaired IKAROS function during recombination of Ig gene segments and peripheral B cell responses may provide a link with the antibody-mediated autoimmune diseases seen in patient II:4 and previously reported cases and with the specific polysaccharide antibody deficiency detected in the asymptomatic mother (I:2), respectively.[2,3] Furthermore, the largely different B cell profile in the here-reported mutant subjects despite similarly low IKAROS protein levels, may suggest the presence of compensatory mechanisms at multiple levels capable of (partially) surmounting the IKAROS defect.[8,9] The actions of modifier genes, epigenetic changes and/or environmental exposures may affect these compensatory mechanisms in different ways, which may, in part, explain the phenotypic heterogeneity in IKAROS-haploinsufficient patients.[8,9] Disease-influencing mechanisms in *IKZF1*-associated immunodeficiency await further study.

In conclusion, we report on the first truncating *IKZF1* mutation associated with IKAROS haploinsufficiency and illustrate an unexpectedly late and variable block in central and

peripheral B cell development in two patients and their asymptomatic mother. Given the observed immunological abnormalities we recommend close follow up of asymptomatic *IKZF1* mutation carriers.

References

1. John LB, Ward AC. The Ikaros gene family: transcriptional regulators of hematopoiesis and immunity. Mol Immunol 2011;48:1272-8. doi: 10.1016/j.molimm.2011.03.006.
2. Kuehn HS, Boisson B, Cunningham-Rundles C, Reichenbach J, Stray-Pedersen A, Gelfand EW, et al. Loss of B cells in patients with heterozygous mutations in IKAROS. N Engl J Med 2016;374:1032-43. doi: 10.1056/NEJMoa1512234.
3. Hoshino A, Okada S, Yoshida K, Nishida N, Okuno Y, Ueno H, et al. Abnormal hematopoiesis and autoimmunity in human subjects with germline IKZF1 mutations. J Allergy Clin Immunol 2016. pii: S0091-6749(16)31273-8. [Epub ahead of print]. doi: 10.1016/j.jaci.2016.09.029.
4. Goldman FD, Gurel Z, Al-Zubeidi D, Fried AJ, Icardi M, Song C, et al. Congenital pancytopenia and absence of B lymphocytes in a neonate with a mutation in the Ikaros gene. Pediatr Blood Cancer 2012;58:591-7. doi: 10.1002/pbc.23160.
5. Kakinuma S, Kodama Y, Amasaki Y, Yi S, Tokairin Y, Arai M, et al. Ikaros is a mutational target for lymphomagenesis in Mlh1-deficient mice. Oncogene 2007;26:2945-9. doi: 10.1038/sj.onc.1210100.
6. Bogaert DJ, Dullaers M, Lambrecht BN, Vermaelen KY, De Baere E, Haerynck F. Genes associated with common variable immunodeficiency: one diagnosis to rule them all? J Med Genet 2016;53:575-90. doi: 10.1136/jmedgenet-2015-103690.
7. Spinner MA, Sanchez LA, Hsu AP, Shaw PA, Zerbe CS, Calvo KR, et al. GATA2 deficiency: a protean disorder of hematopoiesis, lymphatics, and immunity. Blood 2014;123:809-21. doi: 10.1182/blood-2013-07-515528.
8. Sellars M, Kastner P, Chan S. Ikaros in B cell development and function. World J Biol Chem 2011;2:132-9. doi: 10.4331/wjbc.v2.i6.132.
9. Schjerven H, McLaughlin J, Arenzana TL, Frietze S, Cheng D, Wadsworth SE, et al. Selective regulation of lymphopoiesis and leukemogenesis by individual zinc fingers of Ikaros. Nat Immunol 2013;14:1073-83. doi: 10.1038/ni.2707.

Acknowledgements

The authors gratefully acknowledge the patients and their family who participated in this study. We thank Veronique Debacker, Nancy De Cabooter and Kelly Heyns for excellent technical assistance.

This study was supported by the Ghent University Hospital Spearhead Initiative for Immunology Research, the Jeffrey Modell Foundation to F.H., the Research Foundation Flanders (FWO) to D.B. and E.D.B., the Wellcome Trust to U.C. and V.B., the Ghent University Special Research Fund (BOF15/GOA/011) to E.D.B., Hercules foundation AUGE/13/023 to E.D.B., and the NIH Clinical Center intramural research program to H.S.K., K.R.C. and S.D.R. D.B. is a PhD fellow and E.D.B. a Senior Clinical Investigator of the FWO.

Supplementary Information

SUPPLEMENTARY CLINICAL INFORMATION

The first two pregnancies in the index family were complicated by premature labor of unknown etiology at 25 weeks (II:1) and 15 weeks (II:2) gestational age, respectively. Both children were not viable. The three remaining sisters (II:3, II:4, II:5) were born around 35 weeks gestational age and had an uneventful postnatal period.

SUPPLEMENTARY METHODS

Ethics

All reported subjects provided written informed consent for participation in the study, in accordance with the 1975 Helsinki Declaration. The research protocol was approved by the ethical committee of Ghent University Hospital (2012/593).

Whole exome sequencing (WES)

WES was performed in the two affected siblings (II:3, II:4) and both parents (I:1, I:2). Genomic DNA was isolated from whole blood leukocytes using the Puregene DNA isolation kit (QIAGEN) according to manufacturer's instructions. Whole exome enrichment was performed with the SureSelectXT Human All Exon V6 kit (Agilent Technologies). Paired-end massively parallel sequencing (100 cycles) was performed on a NextSeq 500 (Illumina). Read mapping against the human genome reference sequence (NCBI, GRCh37), and post-mapping duplicate read removal, quality-based variant calling and coverage analysis were performed with CLC Genomics Workbench v6.0.4 (CLC bio). More than 98% of enriched regions had a read depth of at least 20x. Called variants with coverage ≥ 3 were annotated with Alamut Batch (Interactive Biosoftware). Only variants with population frequencies less than 10% were considered, according to public databases NCBI dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), NHLBI Exome Sequencing Project - Exome Variant Server (http://evs.gs.washington.edu/EVS/), ExAC Browser (http://exac.broadinstitute.org/), and 1000 Genomes Project Browser (http://browser.1000genomes.org/). Variants were further prioritized based on allele frequency, functional prediction scores, nucleotide conservation scores and biological relevance.[E1] Both Mendelian and non-Mendelian inheritance patterns were taken into account. Afterwards, variants of interest were evaluated using Alamut Visual mutation interpretation software v2.7 rev. 1 (Interactive Biosoftware), Ingenuity Variant Analysis

(QIAGEN, 2015 Release Spring), CADD scores v1.3 (http://cadd.gs.washington.edu/home), genome Aggregation Database (gnomAD) Browser (http://gnomad.broadinstitute.org), literature search, segregation analysis in available family members, and frequency in an in-house database containing variants of more than 1000 exomes at time of analysis.

Sanger sequencing of genomic DNA

The DNA template (GRCh37/hg19) of *IKZF1* (NM_006060) was obtained from UCSC Genome Browser (https://genome.ucsc.edu). Primers for amplification and sequencing of exon 3 and adjacent intron-exon boundaries were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)[E2]: forward primer 5'-CTCATGCCACCCTCTCAAG-3' and reverse primer 5'-GAGTGTCCATCCTCCCACAC-3'. Genomic DNA was amplified by PCR using the specific primers and KAPA2G Robust Hotstart Ready Mix (KAPA Biosystems). PCR products were enzymatically purified with Exonuclease I and Antarctic phosphatase (both New England BioLabs Inc.). Purified PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on a 3730xl DNA Analyzer (Applied Biosystems). Sequence reads were analyzed with SeqScape v2.5 (ThermoFisher Scientific).

Sanger sequencing of complementary DNA (cDNA)

Total RNA was isolated from peripheral blood mononuclear cells (PBMCs) of all available family members (I:1, I:2, II:3, II:4, II:5) and two healthy controls by use of the RNeasy Plus Mini Kit (QIAGEN) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad), according to manufacturer's instructions. The cDNA template (GRCh37/hg19) of *IKZF1* (NM_006060) was obtained from Ensembl Genome Browser (http://www.ensembl.org/index.html). Primers for amplification and sequencing of exon 3 and adjacent coding regions were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)[E2]: forward primer 5'-ATGGATGCTGATGAGGGTCAAG-3' and reverse primer 5'-CGGAATGCAGCTTGATGTGCAGGAGC-3'. PCR amplification was performed by use of GoTaq Hot Start Colorless Master Mix (Promega). Purified PCR products were Sanger sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on a 3130xL Genetic Analyzer (Applied Biosystems). Sequence reads were analyzed with SeqMan (DNAStar).

***IKZF1* gene expression**

Total RNA was isolated from PBMCs of two mutant family members (II:3, II:4) and three healthy controls by use of the RNeasy Plus Mini Kit (QIAGEN) and converted to cDNA using

the Transcriptor High Fidelity cDNA Synthesis Kit (Roche), according to manufacturer's instructions. Two amplicons of *IKZF1* were investigated: amplicon 1 located upstream of the mutation at genomic position Chr7:50358652-50367269, and amplicon 2 located downstream of the mutation at genomic position Chr7:50469628-50469734 (GRCh37/hg19). These amplicons were chosen to target as many *IKZF1* transcripts as possible. Primers were designed using PrimerXL and IDT primerquest (available upon request). *GPI* and *PSMB2* were used as reference genes. Gene expression was analyzed by real-time quantitative PCR using LightCycler 480 SYBR Green I Master reagent and a LightCycler 96 Instrument (both Roche) according to manufacturer's instructions. All reactions were performed in triplicate. The relative quantification of gene expression was calculated with the comparative Ct method.

Flow cytometric analysis of peripheral blood B and T cell subsets

Peripheral blood B and T cell subsets were evaluated in the *IKZF1* mutation carriers (I:2, II:3, II:4) and age-matched healthy controls as previously described.[E3] In brief, cryopreserved PBMCs were stained with fixable viability dye 506 (eBioscience) and fluorescently labeled monoclonal antibodies under saturation conditions. Following antibodies (clones) were used: CD8 (RPA-T8), CD16 (3G8), CD19 (HIB19), CD20 (2H7), CD21 (B-LY4), CD27 (M-T271), CXCR5 (RF8B2), IgD (IA6-2), γδTCR (11F2) (all BD Biosciences); CD3 (SK7), CD4 (SK3), CD24 (ML5), CD25 (BC96), CD45RO (UCHL1), CCR7 (G043H7) (all Biolegend); CD4 (RPA-T4), CD38 (HIT2), CD56 (TULY56), Foxp3 (PCH101) (all eBioscience). Cells were acquired on an LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo version X (Tree Star Inc.).

IKAROS intracellular staining

Cryopreserved PBMCs were first stained with fluorescently labeled anti-CD3 (clone UCHT1) and anti-CD19 (clone SJ25C1) (both BD Biosciences). Afterwards, cells were fixed and permeabilized using FoxP3 staining kit (eBioscience) following manufacturer's instructions, and incubated with fluorescently labeled anti-IKAROS (clone R32-1149, BD Biosciences).[E4] Cells were acquired on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo version X (Tree Star Inc.).

Flow cytometric analysis of bone marrow aspirates

Bone marrow aspirates from the hipbone were obtained from subjects I:2 and II:3, with informed consent and the ethical approval of Newcastle and North Tyneside Research Ethics Committee.

Analysis of hematopoietic stem and lineage progenitor cells was based on Doulatov *et al*.[E5] The following antibodies (clones) were used: CD3 (SK7-Leu9), CD19 (HIB19), CD20 (L27), CD56 (NCAM16.2), CD38 (HB7), CD45RA (HI100), CD90 (G20-127), CD10 (HI10A) (all BD Biosciences), and CD34 (581) (Biolegend). Cells were acquired on an LSR Fortessa X-20 flow cytometer (BD Biosciences), with dead cell exclusion by DAPI (Sysmex).

For analysis of B-lineage cells, cryopreserved bone marrow samples were stained as previously described.[E6] The following antibodies (clones) were used: CD5 (L17F12), CD10 (HI10a), CD19 (SJ25C1), CD20 (2H7), CD45 (2D1), CD34 (8G12) (all BD Biosciences). Samples were acquired on a FACSCanto II flow cytometer (BD Biosciences).

All data were analyzed with FlowJo version X (Tree Star Inc.).

Statistical analysis

IKZF1 gene expression and IKAROS expression levels were compared using the Mann-Whitney U test, with GraphPad Prism Software v7. A two-sided p value ≤ 0.05 was considered statistically significant.

SUPPLEMENTARY TABLE

Table E1. Routine immunological laboratory evaluation.

	Patient II:3		Patient II:4		Patient I:2	
	Value	**Reference range**	**Value**	**Reference range**	**Value**	**Reference range**
White blood cells	*Age: 13 years*		*Age: 11 years*		*Age: 42 years*	
Total leukocytes (no./µL)	6820	4500-12000	5050	4500-12000	11320	3650-9300
Neutrophils (no./µL)	3580	2500-8000	2240	2500-8000	7850	1573-6100
Lymphocytes (no./µL)	1690	1500-6500	2030	1500-6500	2350	1133-3105
CD3+ T cells (no./µL)	1370	800-3500	1520	800-3500	1930	700-2100
CD3+CD4+ T helper cells (no./µL)	659	400-2100	771	400-2100	1030	300-1400
CD45RA+ naive CD4+ T cells (%)	32	33-66 †	52	46-77 †	24	NA
CD45RO+ memory CD4+ T cells (%)	60	18-38 †	34	13-30 †	72	NA
CD3+CD8+ T cytotoxic cells (no./µL)	575	200-1200	508	200-1200	729	200-1200
CD45RA+ naive CD8+ T cells (%)	63	61-91 †	80	63-92†	47	NA
CD45RO+ memory CD8+ T cells (%)	32	4-23 †	16	4-21 †	53	NA
Ratio CD4/CD8	1.15	0.9-3.4	1.52	0.9-3.4	1.42	1.0-3.6
CD19+ B cells (no./µL)	17	200-600	122	200-600	141	100-500
IgD+CD27- naive B cells (%)	93	51.3-82.5 ‡	90	51.3-82.5 ‡	77	48.4-79.7 ‡
CD24++CD38++ transitional B cells (%)	50	1.4-13.0 ‡	3	1.4-13.0 ‡	1	0.9-5.7 ‡
IgD-CD27+ switched memory B cells (%)	1.5	8.7-25.6 ‡	3	8.7-25.6 ‡	10	8.3-27.8 ‡
IgD+CD27+ marginal zone B cells (%)	2	4.6-18.2 ‡	2	4.6-18.2 ‡	9	7.0-23.8 ‡
$CD21^{low}$ $CD38^{low}$ B cells (%)	3	2.7-8.7 ‡	3	2.7-8.7 ‡	3	1.6-10.0 ‡
CD3-CD56+CD16+ NK cells (no./µL)	304	70-1200	386	70-1200	259	90-600
Monocytes (no./µL)	730	500-1000	650	500-1000	820	247-757
Eosinophils (no./µL)	300	100-500	20	100-500	240	28-273
Basophils (no./µL)	30	10-100	80	10-100	30	6-50
Immunoglobulins *	*Age: 9 years*		*Age: 11 years*		*Age: 42 years*	
IgG (g/L)	7.0	4.70-11.9	12.7	4.70-11.9	10.7	7.0-16.0
IgG2 (g/L)	1.3	0.98-4.8	3.69	0.98-4.8	2.09	1.50-6.40
IgG3 (g/L)	0.668	0.15-1.49	0.309	0.15-1.49	0.617	0.20-1.10
IgM (g/L)	Undetectable	0.27-0.74	0.4	0.27-0.74	0.35	0.40-2.48
IgA (g/L)	Undetectable	0.50-1.66	Undetectable	0.50-1.66	0.98	0.71-3.65
IgE (kU/L)	< 4.4	0-90	44.2	0-90	< 4.4	0-100
Specific antibody responses *	*Age: 9 years*		*Ages: 5 and 7 years*		*Age: 42 years*	
S. pneumoniae polysaccharide IgG (Lab U)	< 3	≥ 11: immune	3	≥ 11: immune	NA	
S. pneumoniae polysaccharide IgG : specific IgG response to 3 serotypes (8, 9N, 15B)	NA		Insufficient antibody responses	Adequate titer increase for at least 2 out of 3 serotypes $	Borderline insufficient antibody responses	Adequate titer increase for at least 2 out of 3 serotypes $
Tetanus IgG (IU/mL)	1.0	≥ 0.01: immune	0.3	≥ 0.01: immune	0.01	≥ 0.01: immune
Rubella IgG (IU/mL)	18	> 10: immune	68	> 10: immune	NA	
Measles IgG (mIU/mL)	610	> 300: immune	1000	> 300: immune	NA	
Mumps IgG (Lab U/mL)	1800	> 500: immune	< 230	> 500: immune	NA	
Varicella Zoster IgG (mIU/mL)	580	> 100: immune	1900	> 100: immune	NA	
Lymphocyte proliferation assay	*Age: 9 years*		*Age: 5 years*			
Response to Concanavalin A	Normal	Compared to control	Normal	Compared to control	NA	
Response to Phytohemagglutinin	Normal	Compared to control	Normal	Compared to control	NA	
Response to Tetanus toxoid	Moderately reduced	Compared to control	Normal	Compared to control	NA	

The most recent, comprehensive and representative laboratory results are shown for each patient, with patient age at time of analysis mentioned. Patients II:3 and II:4 were immunized according to the recommended childhood immunization schedule that, among others, included tetanus, measles, mumps, rubella and 7-valent conjugated pneumococcal vaccines. Patient I:2 had received a tetanus booster vaccine within the last 10 years. A polysaccharide (unconjugated) pneumococcal vaccine was given to all three patients at time of immunological evaluation; patient I:2 had never received a pneumococcal vaccine before then. Patients II:3 and II:4 were not vaccinated against varicella zoster virus but had chickenpox in early childhood. NA: not available. *Measured before start of immunoglobulin replacement therapy. †Reference values from Shearer *et al.*[E7] ‡Reference values from Piatosa *et al.*[E8] $According to the recommendations of Orange *et al.*[E9]

SUPPLEMENTARY FIGURES

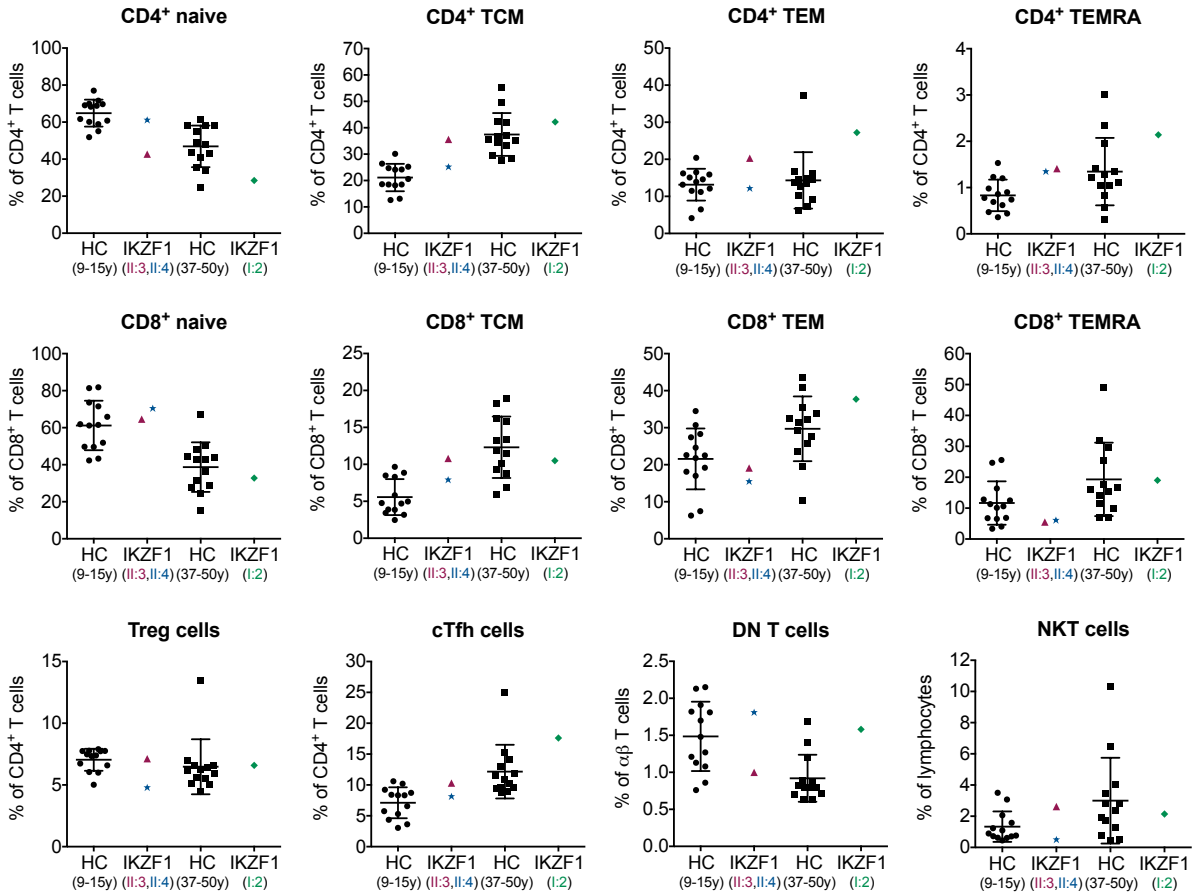


Figure E1. Peripheral blood T cell subsets. Flow cytometric immunophenotyping of T cell subsets was performed on patients' peripheral blood mononuclear cells (PBMCs) in comparison with age-matched healthy controls (HC). At time of analysis, patients I:2, II:3, and II:4 were 40, 14, and 9.5 years old, respectively. Patient II:3 is indicated as purple triangle, patient II:4 as blue star. Total T cells were gated as $CD3^+$ in alive PBMCs. αβ T cells were gated as $\gamma\delta TCR^-$ in total T cells. $CD4^+$ and $CD8^+$ T cells were gated in αβ T cells. In $CD4^+$ and $CD8^+$ T cells, naive cells were gated as $CD45RO^-CCR7^+$, central memory cells (TCM) as $CD45RO^+CCR7^+$, effector memory cells (TEM) as $CD45RO^+CCR7^-$, and terminally differentiated cells (TEMRA) as $CD45RO^-CCR7^-$. Regulatory T (Treg) cells were gated as $CD25^+Foxp3^+$ and circulating follicular helper T (cTfh) cells as $CXCR5^+CD45RO^+$ in $CD4^+$ T cells. Double negative (DN) T cells were gated as $CD4^-CD8^-$ in αβ T cells. Natural killer (NK) T cells were gated as $CD56^+$ in total $CD3^+$ T cells.

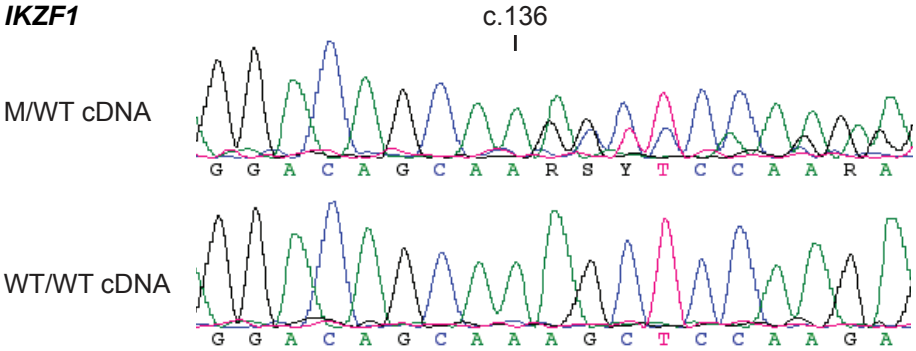


Figure E2. *IKZF1* cDNA sequencing. Representative *IKZF1* cDNA sequences of a mutant (M/WT) and wild type (WT/WT) family member. cDNA was derived from total peripheral blood mononuclear cells. The position of the nucleotide deletion (c.136) is shown on top. The M/WT individual has both a mutant and a wild type cDNA sequence, suggesting that the mutant transcript escapes nonsense-mediated mRNA decay.

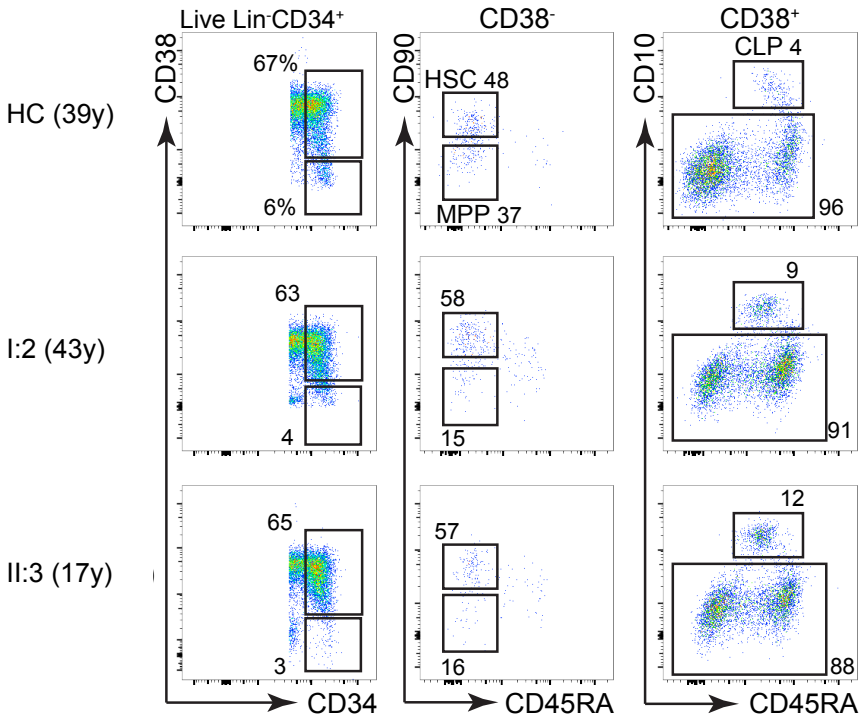


Figure E3. Flow cytometric analysis of hematopoietic stem and early lineage progenitor cells in the bone marrow. The gating strategy was based on Hoshino *et al.*[E10] Percentages represent percentage of parent population, indicated on top of each plot. CLP: common lymphoid progenitor, HSC: hematopoietic stem cell, MPP: multipotent progenitor, y: years.

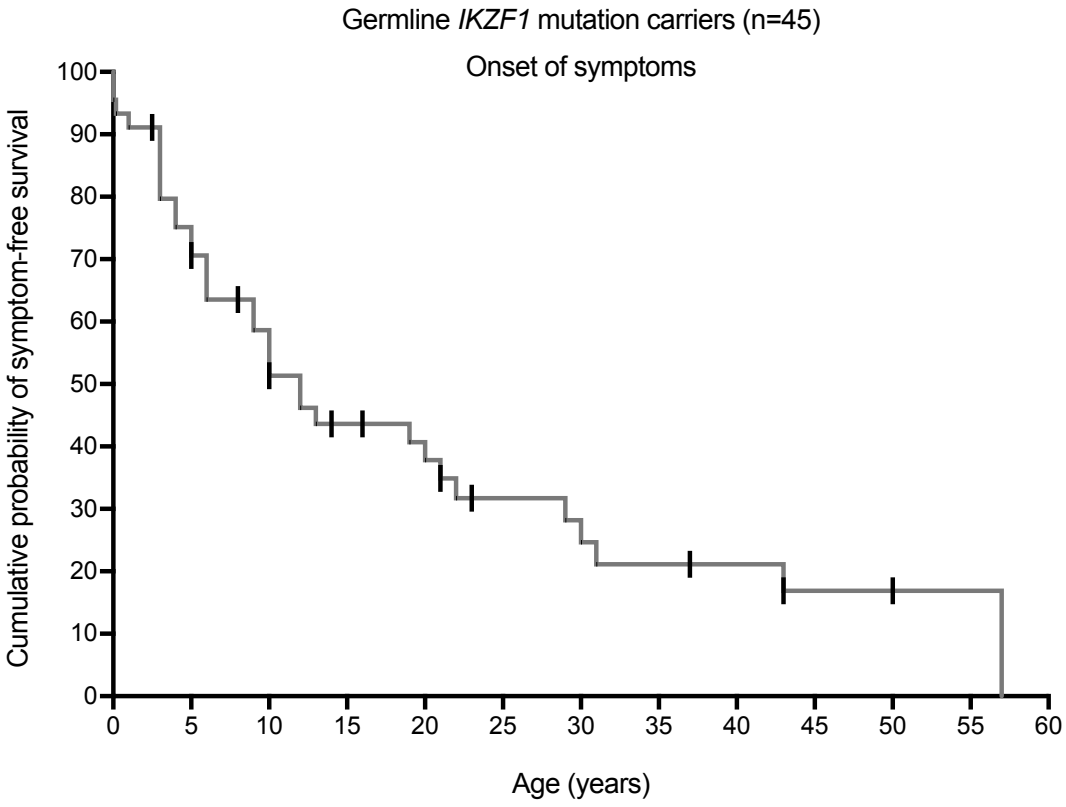


Figure E4. Kaplan-Meier curve on symptom-free survival in germline *IKZF1* mutation carriers. The curve was generated based on the presence and the age of onset of clinical symptoms in the 42 previously published cases and the 3 here-reported cases with germline heterozygous *IKZF1* mutations.[E4,E10,E11] Manifesting subjects first presented with symptoms up to the age of 57 years. Asymptomatic cases were 50 years or younger at time of publication (i.e. censored subjects, indicated on the curve as black tick marks). The Kaplan-Meier symptom-free survival curve estimates that all *IKZF1* mutation carriers will have developed symptoms by 57 years of age.

SUPPLEMENTARY REFERENCES

E1. Coppieters F, Van Schil K, Bauwens M, Verdin H, De Jaegher A, Syx D, et al. Identity-by-descent-guided mutation analysis and exome sequencing in consanguineous families reveals unusual clinical and molecular findings in retinal dystrophy. Genet Med 2014;16(9):671-680. doi: 10.1038/gim.2014.24.

E2. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 2007;35:W71-74. doi: 10.1093/nar/gkm306.

E3. Bogaert DJ, De Bruyne M, Debacker V, Depuydt P, De Preter K, Bonroy C, et al. The immunophenotypical fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. Haematologica. 2017;102(1):192-202. doi: 10.3324/haematol.2016.149112.

E4. Kuehn HS, Boisson B, Cunningham-Rundles C, Reichenbach J, Stray-Pedersen A, Gelfand EW, et al. Loss of B cells in patients with heterozygous mutations in IKAROS. N Engl J Med. 2016;374(11):1032-43. doi: 10.1056/NEJMoa1512234.

E5. Doulatov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, Dick JE. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nat Immunol. 2010;11(7):585-93.

E6. Ganapathi KA, Townsley DM, Hsu AP, Arthur DC, Zerbe CS, Cuellar-Rodriguez J, et al. GATA2 deficiency-associated bone marrow disorder differs from idiopathic aplastic anemia. Blood. 2015;125(1):56-70. doi: 10.1182/blood-2014-06-580340.

E7. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol. 2003;112(5):973-80.

E8. Piatosa B, Wolska-Kusnierz B, Pac M, Siewiera K, Galkowska E, Bernatowska E. B cell subsets in healthy children: reference values for evaluation of B cell maturation process in peripheral blood. Cytometry B Clin Cytom. 2010;78(6):372-81. doi: 10.1002/cyto.b.20536.

E9. Orange JS, Ballow M, Stiehm ER, Ballas ZK, Chinen J, De La Morena M, et al. Use and interpretation of diagnostic vaccination in primary immunodeficiency: a working group report of the Basic and Clinical Immunology Interest Section of the American Academy of Allergy, Asthma & Immunology. J Allergy Clin Immunol. 2012;130(3 Suppl):S1-24. doi: 10.1016/j.jaci.2012.07.002.

E10. Hoshino A, Okada S, Yoshida K, Nishida N, Okuno Y, Ueno H, et al. Abnormal hematopoiesis and autoimmunity in human subjects with germline IKZF1 mutations. J Allergy Clin Immunol. 2016 Dec 1. pii: S0091-6749(16)31273-8. doi: 10.1016/j.jaci.2016.09.029. [Epub ahead of print]

E11. Goldman FD, Gurel Z, Al-Zubeidi D, Fried AJ, Icardi M, Song C, et al. Congenital pancytopenia and absence of B lymphocytes in a neonate with a mutation in the Ikaros gene. Pediatr Blood Cancer. 2012;58(4):591-7. doi: 10.1002/pbc.23160.

Chapter 7

Compound heterozygous *GTF3A* mutations cause a novel ribosomopathy characterized by CVID-like primary immunodeficiency disease

7 COMPOUND HETEROZYGOUS *GTF3A* MUTATIONS CAUSE A NOVEL RIBOSOMOPATHY CHARACTERIZED BY CVID-LIKE PRIMARY IMMUNODEFICIENCY DISEASE

Delfien J.A. Bogaert[1,2,3,4,5], Hye Sun Kuehn[6], Tessa Kerre[4,7,8,9], Carolien Bonroy[4,10], Marieke De Bruyne[1,2,3,4], Julie E. Niemela[6], Frans De Baets[2,4], Myriam Van Winckel[11], Thomas A. Fleisher[6], Sergio D. Rosenzweig[6], Elfride De Baere[3,4], Melissa Dullaers[1,4,5,8,*], Filomeen Haerynck[1,2,4,*]

[1]Clinical Immunology Research Lab, Department of Pulmonary Medicine, Ghent University Hospital, Ghent, Belgium; [2]Department of Pediatrics, Division of Pediatric Immunology and Pulmonology, Ghent University Hospital, Ghent, Belgium; [3]Center for Medical Genetics, Ghent University and Ghent University Hospital, Ghent, Belgium; [4]Centre for Primary Immunodeficiency, Jeffrey Modell Diagnosis and Research Centre, Ghent University Hospital, Ghent, Belgium; [5]Laboratory of Immunoregulation, VIB Inflammation Research Center, Ghent, Belgium; [6]Immunology Service, Department of Laboratory Medicine, NIH Clinical Center, National Institutes of Health, Bethesda, MD, USA; [7]Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Ghent, Belgium; [8]Department of Internal Medicine, Ghent University, Ghent, Belgium; [9]Department of Hematology, Ghent University Hospital, Ghent, Belgium; [10]Department of Laboratory Medicine, Ghent University Hospital, Ghent, Belgium; [11]Department of Pediatrics, Division of Pediatric Gastroenterology, Hepatology and Nutrition, Ghent University Hospital, Ghent, Belgium; *MD and FH contributed equally to this work.

Manuscript in preparation.

ABSTRACT

In two Belgian siblings with a CVID-like phenotype born to non-consanguineous parents, we used whole exome sequencing to identify novel compound heterozygous missense variants in *General Transcription Factor IIIA* (*GTF3A*), segregating with disease and predicted to be pathogenic. The consequences of *GTF3A* mutations in humans or mice are unknown, and a specific role of *GTF3A* in the immune system has so far not been described. Transcription factor IIIA (TFIIIA), encoded by *GTF3A*, plays an indispensable role in transcription regulation of 5S ribosomal RNA (rRNA) encoding genes. 5S rRNA is a component of ribosomes, responsible for protein synthesis. TFIIIA depletion in human cell lines reduces 5S rRNA production and impairs ribosome biogenesis. Ribosomopathies, diseases caused by inborn defects in ribosome biogenesis, give rise to cell- and tissue-specific deficits and have been associated with immunological abnormalities reminiscent of CVID. In the here-reported patients, 5S rRNA expression was significantly reduced compared to the unaffected mother and healthy controls. Furthermore, both patients had impaired B cell and (albeit less-pronounced) T cell proliferation, which is compelling because 5S rRNA and ribosome biogenesis have important functions in cell cycle regulation and B and T cell proliferation defects have been previously documented in ribosomopathies. Altogether, we hypothesize that biallelic loss-of-function *GTF3A* mutations may cause a novel ribosomopathy associated with CVID-like primary immunodeficiency disease. Future studies are planned to investigate the causal relationship between the *GTF3A* genotype and the clinical phenotype via relevant cellular models.

INTRODUCTION

Common variable immunodeficiency (CVID) is one of the most frequently diagnosed primary immunodeficiencies (PIDs) defined as a marked decrease in serum IgG, decreased IgM and/or IgA, impaired production of specific antibodies after immunization, and exclusion of defined causes of hypogammaglobulinemia.[1,2] CVID encompasses a phenotypically and genetically heterogeneous group of patients characterized by an increased susceptibility to (sinopulmonary) infections and features of immune dysregulation such as autoimmunity and lymphoproliferative disease.[1] Furthermore, there are a vast number of patients with a clinical and laboratory phenotype reminiscent of CVID but not fulfilling all above-mentioned diagnostic criteria.[1] In the past years, next-generation sequencing (NGS) technologies have facilitated the discovery of novel disease genes in patients diagnosed with CVID or CVID-like disease.[3] These genetic discoveries have not only advanced our understanding of disease pathogenesis, but also uncovered genes with a previously unknown function in immunity (e.g. *LRBA*).[3]

Here, we report a brother and sister pair presenting with a CVID-like phenotype in early childhood, in whom we identified novel compound heterozygous variants in *General Transcription Factor IIIA* (*GTF3A*) as possible underlying genetic cause. To our knowledge, *GTF3A* has not yet been associated with human disease. *GTF3A* encodes transcription factor IIIA (TFIIIA), which is a ubiquitously expressed Cys_2His_2 zinc finger protein.[4] The 365 amino acid sequence of human TFIIIA contains nine Cys_2His_2 zinc finger motifs and a non-finger C-terminal tail.[5] TFIIIA has been extensively studied for its role in transcription regulation of *5S ribosomal DNA (rDNA)* genes, encoding 5S ribosomal RNA (rRNA).[6,7] 5S rRNA is a component of the 60S large ribosomal subunit.[8] The human genome contains multiple *5S rDNA* gene copies organized in clusters of tandem repeats.[9] Binding of TFIIIA to the internal promoter region in the *5S rDNA* genes initiates recruitment of TFIIIC, TFIIIB and RNA polymerase III (Pol III), forming a transcription complex by which the *5S rDNA* genes are ultimately transcribed by Pol III.[7] In addition, TFIIIA acts as a chaperone by binding and stabilizing 5S rRNA as well as other (unspecified) transcripts.[10] Binding between 5S rRNA and TFIIIA, in turn, represses further transcription of the *5S rDNA* genes.[11] Furthermore, Pol III is also known for transcribing other small noncoding RNA genes involved in translation, such as *RNase P*, *7SK RNA* and all transfer RNA (tRNA)-coding genes.[7] Transcription initiation of these genes occurs independently of TFIIIA.[7,8]

The function of *GTF3A* and its product TFIIIA have been mainly studied in model organisms such as *Xenopus laevis* (frog), *Arabidopsis thaliana* (plant) and *Saccharomyces cerevisiae* (yeast). [11-16] Thus far, a specific role of TFIIIA in the immune response is unknown and no mouse models have been reported. Silencing of *GTF3A* in human cell lines suppressed the

production of nascent 5S rRNA and impaired ribosome biogenesis.[8] Defects in ribosome biogenesis form a class of diseases known as ribosomopathies, of which Diamond-Blackfan anemia and Shwachman-Diamond syndrome are typical examples.[17] Ribosomes consist of ribosomal proteins and rRNA that are widely or even ubiquitously expressed in human tissues. Given the universal requirement of ribosomes for protein synthesis, it was long assumed that defective ribosome biogenesis would be incompatible with life.[18] Strikingly, with the identification of genetic defects in ribosomal components, it became clear that ribosomopathies were not lethal but were in fact associated with unique tissue-specific phenotypes.[17] Current knowledge on disease mechanisms in ribosomopathies is limited.[18] The most puzzling question in ribosomopathy-related research is how the disruption of ubiquitously expressed genes involved in fundamental processes can lead to cell- and tissue-specific deficits.[18] One proposed explanation is the existence of cell- and tissue-specific specialized ribosomes that recognize specific mRNAs.[18] Others speculate that there may be spatiotemporal differences in ribosome biogenesis meaning that the required threshold levels of activity may be different in one tissue versus another at different times.[17] Common features among ribosomopathies are bone marrow failure and congenital (mainly skeletal) abnormalities.[17] Considerable phenotypical variability is, however, seen in all ribosomopathies; e.g. about half of patients with Diamond-Blackfan anemia only present with anemia without dysmorphic features.[17] Some, but not all, ribosomopathies increase patients' susceptibility to solid tumors or hematological malignancies.[19] Interestingly, several ribosomopathies have been associated with immunological defects, including reduced numbers and function of both T cells and B cells.[20] Even more, in 2011, Khan *et al* stated that ribosomopathies would be able to explain some patients presenting with a CVID phenotype.[20]

We hypothesize that the here-reported compound heterozygous *GTF3A* variants cause loss of TFIIIA function, impairing 5S rRNA generation and subsequent ribosome biogenesis, and resulting in a novel ribosomopathy characterized by a CVID-like primary immunodeficiency disease. We present preliminary data from the initial validation studies and discuss plans for future research.

METHODS

Ethics

All reported subjects provided written informed consent for participation in the study, in accordance with the 1975 Helsinki Declaration. The research protocol was approved by the ethical committee of Ghent University Hospital (2012/593).

Microarray-based comparative genomic hybridization (array CGH)

Array CGH was performed in both affected siblings (II:1, II:3) using the SurePrint G3 Human CGH Microarray Kit with an average genome-wide resolution of 100 kb, according to manufacturer's instructions (Agilent Technologies). Results were analyzed using arrayCGHbase.[21]

Whole exome sequencing (WES)

WES was performed in the two affected siblings (II:1, II:3) and both parents (I:1, I:2). Genomic DNA was isolated from whole blood leukocytes using the Puregene DNA isolation kit (QIAGEN) according to manufacturer's instructions. Whole exome enrichment was performed with the SureSelectXT Human All Exon V5+UTRs kit (Agilent Technologies). Paired-end massively parallel sequencing (100 cycles) was performed on a NextSeq 500 (Illumina). Read mapping against the human genome reference sequence (NCBI, GRCh37), and post-mapping duplicate read removal, quality-based variant calling and coverage analysis were performed with CLC Genomics Workbench v6.0.4 (CLC bio). Sequencing coverage is summarized in Table 1. Called variants with coverage ≥ 3 were annotated with Alamut Batch (Interactive Biosoftware). Only variants with population frequencies less than 10% were considered, according to public databases NCBI dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), NHLBI Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/), 1000 Genomes Project Browser (http://browser.1000genomes.org/), and ExAC Browser (http://exac.broadinstitute.org/). Variants were further prioritized based on allele frequency, functional prediction scores, nucleotide conservation scores and biological relevance.[22] Both Mendelian and non-Mendelian inheritance patterns were taken into account. Afterwards, variants of interest were evaluated using Alamut Visual mutation interpretation software v2.7 rev. 1 (Interactive Biosoftware), Ingenuity Variant Analysis (QIAGEN, 2015 Release Spring), CADD scores v1.3 (http://cadd.gs.washington.edu/home), genome Aggregation Database (gnomAD) Browser (http://gnomad.broadinstitute.org), literature search, segregation analysis in available family

members, and frequency in an in-house database containing variants of more than 1000 exomes at time of analysis.

Table 1. WES coverage.

Subject	Read depth				
	Average read depth	**% of regions ≥ 5 x**	**% of regions ≥ 10 x**	**% of regions ≥ 20 x**	**% of regions ≥ 40 x**
I:1	69.6 x	99.43%	98.12%	92.74%	72.38%
I:2	98.3 x	99.83%	99.67%	98.78%	90.94%
II:1	57.8 x	98.98%	97.41%	89.34%	61.83%
II:3	57.5 x	99.11%	97.35%	88.50%	62.68%

Read depth was calculated for all regions enriched by the SureSelectXT Human All Exon V5+UTRs kit (Agilent Technologies, CA, USA), using CLC Genomics Workbench v6.0.4 (CLC bio, MA, USA).

Sanger sequencing of *GTF3A*

The DNA template (GRCh37/hg19) of *GTF3A* (NM_002097) was obtained from UCSC Genome Browser (https://genome.ucsc.edu). Primers for amplification and sequencing of exons 6 and 7 and adjacent intron-exon borders were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/)[23]: exon 6 forward primer 5'-TCCTTAAAGGGAGGCACCTT-3' and reverse primer 5'-GGGGTGGCTCAGAGACCT-3'; exon 7 forward primer 5'-CAGGTTTCAGGCACTGAATGT-3' and reverse primer 5'-AGCAGGTGAAAAGAATTCCAAA-3'. PCR was performed on an MJ Research PTC-200 Thermal Cycler (MJ Research Inc.). Genomic DNA was amplified using KAPA2G Robust Hotstart Ready Mix (KAPA Biosystems). PCR products were enzymatically purified with Exonuclease I and Antarctic phosphatase (both New England BioLabs Inc.). Purified PCR products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on a 3730xl DNA Analyzer (Applied Biosystems). Sequence reads were analyzed with SeqScape v2.5 (ThermoFisher Scientific).

Protein modeling of TFIIIA

The 3D protein structure of the target sequence, wild type human TFIIIA (UniProtKB ID Q92664), was predicted by I-TASSER[24] using homology-based modeling based on the template structure, *Xenopus laevis* TFIIIA bound to DNA (PDB ID 1TF6)(target-template sequence identity = 53.8%). The 3D homology model was superimposed onto the template to determine the approximate locations of the TFIIIA mutation sites (Cys195 and Cys219) using UCSF Chimera (v1.11.2).[25]

Immunophenotypic analysis of peripheral blood mononuclear cells (PBMCs)

Immunophenotyping of PBMC subsets was performed in the patients (II:1, II:3), their unaffected parents (I:1, I:2), and age-matched healthy controls as previously described.[26] In brief, PBMCs were isolated from EDTA whole blood by Ficoll-Paque density gradient centrifugation and cryopreserved at -150°C. Thawed PBMCs were stained with fixable viability dye 506 (eBioscience) and fluorescently labeled monoclonal antibodies under saturation conditions: T cells (CD3, CD4, CD8, CD25, CD31, CD40, CD45RO, CD127, γδTCR, FOXP3, HLA-DR, CCR7, CXCR5, ICOS, CD69), B cells (CD19, CD20, CD21, CD24, CD27, CD38, CD138, IgD, IgM, IgA, IgG, TACI, BAFF-R, HLA-DR, CCR7, CXCR5), NK cells (CD16, CD56, TCR Vα24-Jα18 iNKT), monocytes (CD14, CD16), dendritic cells (CD11c, CD123). To analyze ICOS and CD69 upregulation on T cells, PBMCs were stimulated with 1% PHA (Life Technologies) for 72 hours at a density of 1.25 x 10^6 PBMCs/mL in supplemented RPMI medium (Gibco). Cells were acquired on an LSR Fortessa flow cytometer (BD Biosciences). Data were analyzed with FlowJo version X (Tree Star Inc.).

T and B cell proliferation, activation and differentiation

Thawed PBMCs were incubated with CellTrace Violet proliferation dye (1µM; CellTrace Violet Cell Proliferation Kit, Invitrogen) for 20 minutes at 37°C. Afterwards, cells were centrifuged and washed twice with RPMI/10% fetal bovine serum (FBS) medium (Gibco). For T cell proliferation and activation, 1 x 10^5 PBMCs were seeded in a 96-well plate in RPMI/10% FBS and stimulated for 4 days with anti-CD3/CD28 (Dynabeads Human T-Activator, ThermoFisher Scientific) or phytohemagglutinin (PHA; 1 m/mL, Sigma-Aldrich), as indicated in the figures. Unstimulated PBMCs were used as negative control. After 4 days, cells were stained with fluorescently labeled CD4, CD8 and CD25 (all BD Biosciences). Proliferation of $CD4^+$ and $CD8^+$ T cells was assessed by CellTrace Violet dye dilution. T cell activation was evaluated by upregulation of CD25 expression. For B cell proliferation, activation and differentiation, 4 x 10^5 PBMCs were seeded in a 96-well plate in RPMI/10% FBS and stimulated for 4 days with different combinations of $F(ab')_2$ anti-IgM (10 ng/mL, Jackson ImmunoResearch Laboratory), CD40L (200 ng/mL, Enzo Life Sciences), IL-21 (100 ng/mL, Cell Signaling), and CpG ODN2006 (2.5 µM, InvivoGen), as indicated in the figures. PBMCs only treated with $F(ab')_2$ anti-IgM were used as negative control. After 4 days, cells were stained with fluorescently labeled CD19, CD25, CD27, CD38 (all BD Biosciences) and CD3 (Invitrogen). Proliferation of $CD19^+$ B cells was assessed by CellTrace Violet dye dilution. B cell activation was evaluated by upregulation of CD25 expression. B cell differentiation into $CD38^{high}CD27^{high}$ plasmablasts was determined.

To further assess B cell activation, 1.5 x 10^5 thawed PBMCs were cultured separately in a 96-well plate in RPMI/10% FBS in the presence of F(ab')$_2$ anti-IgM (10 ng/mL, Jackson ImmunoResearch Laboratory) and soluble CD40 ligand (CD40L; 500 ng/mL, PeproTech). After 4 days, cells were stained with fluorescently labeled CD19, CD25, CD95 (all BD Biosciences), CD80 (eBioscience) and CD3 (Invitrogen). B cell activation was evaluated by upregulation of CD25, CD95 and CD80 expression. PBMCs only stimulated with F(ab')$_2$ anti-IgM were used as negative control.
Cells were acquired on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo version X (Tree Star Inc.). Fold change in mean fluorescence intensity (MFI) was calculated by dividing the MFI of stimulated cells by the MFI of the corresponding negative control. Proliferation indices were computed as the sum of cells in all generations, including the parental generation, divided by the computed number of original parent cells theoretically present at the start of the experiment, whereby the number of parent cells in each n^{th} division was calculated as: (No. of cells in the n^{th} division)/(2n).[27]

Cytokine secretion

Thawed PBMCs were seeded in a 96-well plate (1 x 10^5/well) in RPMI/10% FBS and stimulated with anti-CD3/CD28 (Dynabeads Human T-Activator, ThermoFisher Scientific) for 24 hours, or lipopolysaccharide (LPS; 100 ng/mL, PeproTech) for 24 hours, or F(ab')$_2$ anti-IgM (10 ng/mL, Jackson ImmunoResearch Laboratory) and CD40L (500 ng/mL, PeproTech) for 4 days, as indicated in the figures. Cell-free supernatants were harvested and stored at -80°C. Cytokines (as indicated in the figures) were measured simultaneously with Fluorokine MAP Human Base Kit A (R&D Systems) using the Luminex 200 System (Luminex Corporation).

BTK and PLCγ2 phosphorylation

Thawed PBMCs were seeded in a 96-well plate (1 x 10^5/well) in RPMI/10% FBS and stained with fluorescently labeled CD19 (BD Biosciences). Cells were then stimulated with F(ab')$_2$ anti-IgM (10 ng/mL, Jackson ImmunoResearch Laboratory) for 10 minutes. Unstimulated PBMCs were used as negative control. To terminate the stimulation response, cells were fixated in BD Cytofix/Cytoperm Buffer (BD Biosciences) for 30 minutes at 4°C in the dark. After washing, intracellular staining was performed with fluorescently labeled pBTK/ITK and pPLCγ2 (both BD Biosciences) in BD Perm/Wash Buffer (BD Biosciences). Cells were incubated for 1 hour at 4°C in the dark, washed three times with BD Perm/Wash Buffer, and acquired on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with

FlowJo version X (Tree Star Inc.). Fold change in MFI was calculated by dividing the MFI of stimulated cells by the MFI of unstimulated cells.

STAT1 and STAT5 phosphorylation

Thawed PBMCs were cultured in a 96-well plate (2 x 10^5/well) in RPMI/10% FBS in the presence of IL-7, IFNα or IFNγ (all 10 ng/mL, PeproTech), and fluorescently labeled CD4 (BD Biosciences) for 20 min. Unstimulated PBMCs were used as negative control. To terminate the stimulation response, cells were fixated in BD Cytofix/Cytoperm Buffer (BD Biosciences) for 30 minutes at 4°C in the dark. After washing, intracellular staining was performed with fluorescently labeled pSTAT1 and pSTAT5 (both BD Biosciences) in BD Perm/Wash Buffer (BD Biosciences). Cells were incubated for 1 hour at 4°C in the dark, washed three times with BD Perm/Wash Buffer, and acquired on a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed with FlowJo version X (Tree Star Inc.).

Western blot

Thawed PBMCs (0.5-1.0 x 10^6) were lysed in 0.5% Triton X-100, 0.5% NP-40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, protease inhibitor cocktail (EMD Millipore or Sigma-Aldrich) and phosphatase inhibitor cocktail (ThermoFisher Scientific or Sigma-Aldrich). For western blot analysis of T cell receptor signaling, thawed PBMCs were stimulated with anti-CD3/CD28 (Dynabeads Human T-Activator, ThermoFisher Scientific) in RPMI/10% FBS for 30 minutes prior to lysis. Lysate protein was quantitated using Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Samples of equal protein concentration were prepared. Approximately 20 µg total protein was separated by 4-12% gradient gels and transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBST) for 1 hour at 21°C. Next, membranes were incubated with primary antibody for 2 hours at 21°C. After washing with TBST for 30 minutes at 21°C, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratory) for an additional hour at 21°C. Membranes were again washed with TBST for 30 minutes at 21°C. HRP substrate (ThermoFisher Scientific) was added to the membranes, which were then subjected to chemiluminscent imaging. For re-probing, membranes were first incubated with stripping buffer (ThermoFisher Scientific) for 15 minutes at 21°C, and then blocked and incubated with primary and secondary antibodies as described. The following primary antibodies were used: PLCγ1, NFκB p65, p-NFκB p65, IκBα, p-IKKα/β, IKKβ, p-ERK1/2, β-actin, β-tubulin (all Cell Signaling), TFIIIA (Abcam), and p-PLCγ1 (Invitrogen).

Wild type (WT) and mutant *GTF3A* plasmids

Human *GTF3A* (NM_002097) cDNA was cloned into CMV driven N-terminal flag-tagged expression vector (pFLAG-CMV-2, Sigma-Aldrich). Wild type (WT) *GTF3A* construct was purchased from Dharmacon. The two mutant *GTF3A* constructs (c.585T>G, p.C195W and c.655T>C, p.C219R) were generated via site directed mutagenesis using AccuPrime Pfx DNA Polymerase (Invitrogen) followed by Dpn I digestion (BioLabs). The primers used to generate the mutant constructs are provided in Table 2. Proper expression of the *GTF3A* plasmids was evaluated in HEK293T cells (Figure 1).

Table 2. Primers for site directed mutagenesis of *GTF3A*.

Primer	Sequence (5'-3')
GTF3A C195W, forward	TATGTATGTCAAAAAGGATG**G**TCCTTTGTGGCAAAAACATG
GTF3A C195W, reverse	CATGTTTTTGCCACAAAGGA**C**CATCCTTTTTGACATACATA
GTF3A C219R, forward	CCCATAAAGAGGAAATACTA**C**GTGAAGTATGCCGGAAAACA
GTF3A C219R, reverse	TGTTTTCCGGCATACTTCAC**G**TAGTATTTCCTCTTTATGGG

Nucleotide changes compared to the wild type sequence are marked in bold.

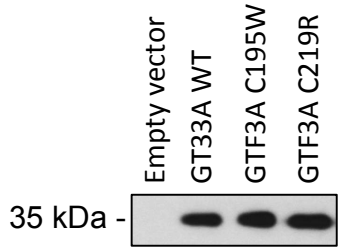


Figure 1. Expression of *GTF3A* plasmids in HEK293T cells. The western blot membrane was probed with anti-flag antibody. Empty flag-tagged vector was used as negative control. TFIIIA protein size is approximately 35 kDa. WT: wild type.

NFκB luciferase reporter assay

HEK293T cells were seeded in a 24-well plate (4 x 10^4/well) in DMEM/10% FBS (Gibco) and transfected with WT, one mutant or both mutant *GTF3A* plasmids along with an NFκB firefly luciferase reporter gene plasmid using Effectene Transfection Reagent (QIAGEN) according to manufacturer's instructions. Each condition was performed in duplicate. Twenty-four hours after transfection, cells were stimulated with TNFα (100 ng/mL, R&D Systems) for 6 hours at 37°C. Next, cells were lysed in 0.5% Triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, and aprotinin protease inhibitor (MP Biomedicals). Luciferase activity was measured using the Luciferase Assay System kit (Promega) on a Synergy 4 Multi-Mode Microplate Reader (BioTek). To verify equal seeding and transfection efficiency, an aliquot of the lysates was prepared for western blot using NuPAGE LDS

Sample Buffer and NuPAGE Sample Reducing Agent (Invitrogen). Western blot for β-actin and Flag was performed as described above.

For each independent experiment, data were normalized by dividing the luciferase signal of the sample by the luciferase signal of unstimulated cells transfected with empty flag-tagged vector (negative control).

Small interfering RNA (siRNA) transfection

siRNA-universal negative control and siRNA-GTF3A were purchased from Sigma-Aldrich. Freshly isolated PBMCs from healthy donors were plated in a 24-well plate (5 x 10^6/well) in RPMI/10% FBS and transfected with 300 nM siRNA using Amaxa electroporation (Lonza) according to manufacturer's instructions. To evaluate transfection efficiency, a separate aliquot of PBMCs was transfected with pmaxGFP provided in the Lonza transfection kit. The next day, transfected cells were washed with fresh RPMI/10% FBS, and cell viability and GFP expression were determined on a FACSCanto II flow cytometer (BD Biosciences). To evaluate B cell proliferation, a first aliquot (1/3) of each sample was incubated the next day with CellTrace Violet proliferation dye as described above, and cultured with $F(ab')_2$ anti-IgM (10 ng/mL, Jackson ImmunoResearch Laboratory) and CD40L (200 ng/mL, Enzo Life Sciences) for an additional period of 4-5 days. Proliferation of $CD19^+$ B cells was assessed by dye dilution as described above. A second aliquot (1/3) of each sample was cultured the next day with anti-IgM and CD40L as described above (without prior labeling with proliferation dye), to determine gene expression at the time of B cell proliferation assessment. A third aliquot (1/3) of each sample was used to determine gene expression at 48 hours after transfection. In some experiments, an additional aliquot of cells was used to evaluate T cell proliferation by means of CellTrace Violet dye dilution at day 4-5 after stimulation with anti-CD3/CD28 (Dynabeads Human T-Activator, ThermoFisher Scientific), as described above.

Gene expression

Total RNA was extracted from unstimulated total PBMCs or siRNA-transfected PBMCs by means of the RNeasy Plus Mini Kit (QIAGEN) according to manufacturer's instructions. Total RNA (100 ng - 1 µg) was reverse transcribed to cDNA using the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Gene expression was analyzed by quantitative PCR (qPCR) using SYBR Green reagent (Applied Biosystems) and StepOnePlus Real-Time PCR System (Applied Biosystems) according to manufacturer's instructions. Expression of the following target genes was investigated: *GTF3A*, *5S rRNA*, *leucyl-tRNA* ($tRNA^{Leu}$), and *tyrosyl-tRNA* ($tRNA^{Tyr}$). *GAPDH* was used as reference gene.

Primers for *5S rRNA*[28], *GAPDH*[28], *tRNA*^{Leu}[29] and *tRNA*^{Tyr}[29] were obtained from previous publications (Table 3). Primers for *GTF3A* were designed in-house with Primer3Plus[23] as described above (Table 3). All reactions were performed in triplicate for 40-45 cycles at Tm 60-65°C. The relative quantification of gene expression was calculated with the comparative Ct method (ΔΔCt method). For each sample, the difference in threshold cycle (Ct) between the target and reference gene was determined as followed: ΔCt = (Ct of target gene) - (Ct of *GAPDH*). Next, data were normalized relative to a chosen control sample. For unstimulated total PBMCs: ΔΔCt = (ΔCt of each sample) - (ΔCt of one healthy control among 2 or 3 healthy controls). For siRNA-transfected PBMCs: ΔΔCt = (ΔCt of each sample) - (ΔCt of the respective donor's control siRNA sample). The fold change in expression of the target gene was calculated as 2^(-ΔΔCt).

Table 3. Primers for qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Primer design
GTF3A	GGAGAAAAGCCGTTTGTTTG	TGGGTGATGCAAAGTGTTTC	In-house
5S rRNA	TACGGCCATACCACCCTGAA	GCGGTCTCCCATCCAAGTAC	Pagano *et al.*[28]
leu-tRNA	ATGGCCGAGTGGTCTAAGG	ACCAGAAGACCCGAACACAG	Birch *et al.*[29]
tyr-tRNA	CCTTCGATAGCTCAGCTGGT	CGACCTAAGGATGTCCACAAAT	Birch *et al.*[29]
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	Pagano *et al.*[28]

Statistical analysis

When indicated, statistical analysis was performed with GraphPad Prism Software v6. A two-sided p value < 0.05 was considered statistically significant.

RESULTS

Case studies

We studied a sister and brother pair from a Belgian, non-consanguineous family (Figure 2A, II:1 and II:3 respectively) who presented with recurrent infections in early childhood. The sister (II:1), currently 28 years old, has been suffering from recurrent upper and lower respiratory tract infections often needing antibiotics since the age of 2 months. She had two severe pneumonias, at 4 and 5 years of age, of which one was complicated with sepsis. At 5 years, she had acute *Campylobacter* gastroenteritis. At 6 years, she was hospitalized for acute *Salmonella* gastroenteritis. Around 10 years of age, she suffered from a persistent intestinal *Giardia lamblia* infection, requiring repeated courses of metronidazole. Immunological lab assessment revealed decreased IgM, normal IgA, normal total IgG and subclasses, and absent global antibody responses to polysaccharide pneumococcal vaccine (Table 4). B and T lymphocyte counts were normal (Figure 3). In accordance with the consensus ESID/PAGID diagnostic criteria at that time, she was diagnosed with possible CVID.[30] During her teenage years, she was doing clinically well with few infections. Since young adulthood, however, she has developed additional symptoms of recurrent lower urinary tract infections, recurrent diarrhea, and fatigue. She started with immunoglobulin replacement therapy at 18 years of age, albeit with variable compliance. At 24 years, she presented with bilateral blue toe syndrome. Biopsy was suggestive of thrombotic vasculopathy. Diagnostic workup for thrombotic disorders including autoimmune vasculitis was, however, negative. At 26 years of age, she gave birth to healthy twin girls. The pregnancy was complicated by preeclampsia at 38 weeks gestational age, requiring an urgent caesarian section. Furthermore, she developed group A Streptococcus postpartum endometritis, responding well to oral antibiotic treatment. Recent immunological lab evaluations suggested a progressive decrease in total B cells (Figure 3). Peripheral B cell subsets, first evaluated at 24 years, showed an abnormal distribution with elevated transitional B cells and high-normal switched memory B cells (Table 4). Total IgG levels have so far remained within normal ranges, meaning that patient II:1 does not fulfill the current consensus diagnostic criteria for CVID.[1]

The younger brother (II:3), currently 18 years of age, had a more severe clinical course. Similar to his sister, patient II:3 has been suffering from recurrent (mainly upper) respiratory tract infections since infancy, often requiring antibiotics. At the age of 7 months, he developed chronic secretory diarrhea with malabsorption. At that time serum IgG, IgA and IgM were decreased, at least partially due to enteric protein loss, and monthly intravenous immunoglobulin therapy was started.

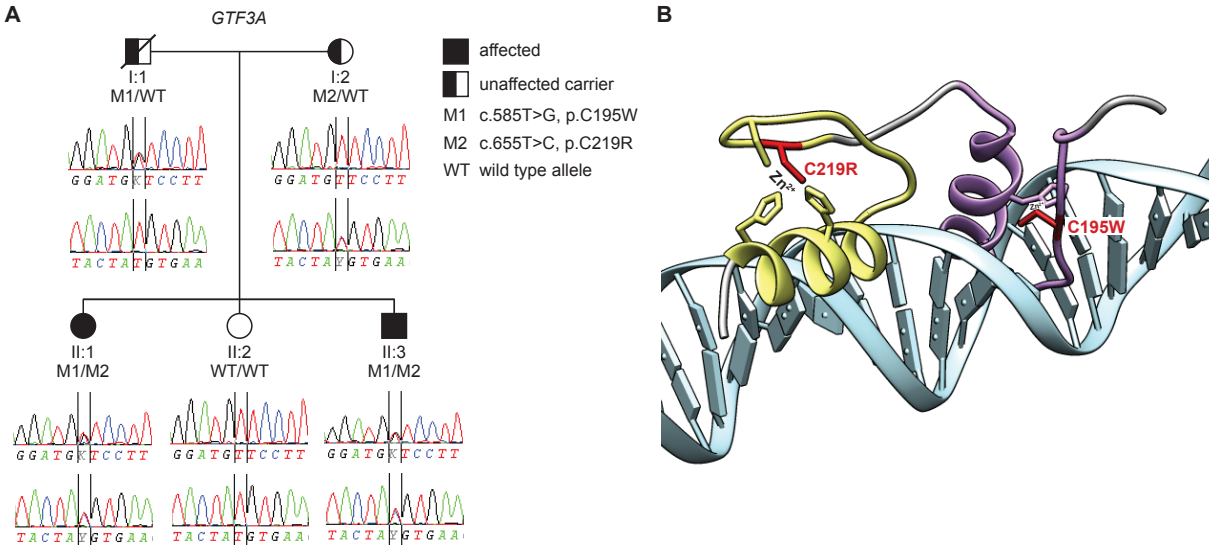


Figure 2. Pedigree and *GTF3A* variants. (A) Pedigree of the core family and corresponding electropherograms. Squares, circles, and diagonal lines indicate males, females and deceased subjects, respectively. **(B)** Predicted 3D model of human wild type TFIIIA zinc finger 6 (purple) and 7 (yellow) bound to DNA (blue). The mutation sites at two different conserved cysteines (C195 & C219) are highlighted in red. As these conserved residues act within a Cys_2His_2 zinc finger motif to coordinate zinc ions (Zn^{2+}) that stabilize the fold, this model suggests that both mutations would destabilize the structure, thereby disrupting DNA binding. For clarity, only one conserved cysteine (stick) and histidine (pentagon) is shown for zinc finger 6.

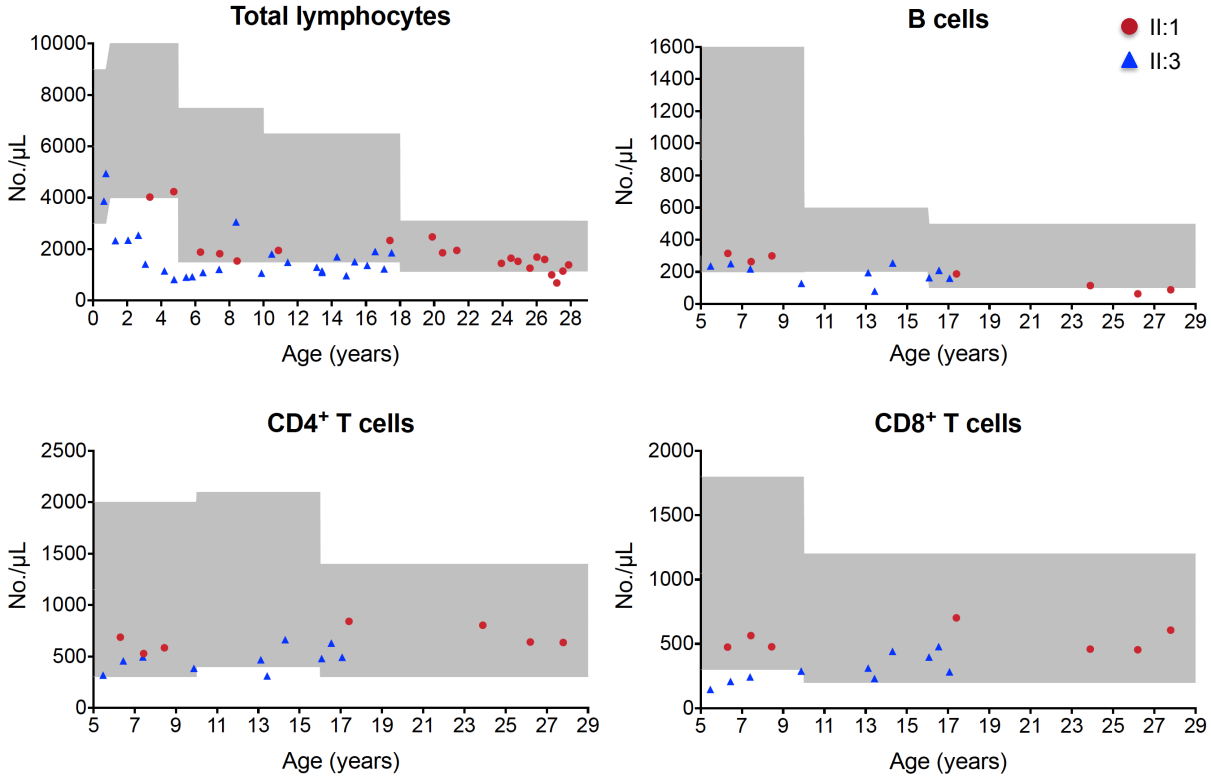


Figure 3. Evolution of peripheral blood lymphocyte counts in the patients. The most comprehensive and representative laboratory evaluations performed in our center are shown. Grey shadings indicate the age-based reference ranges.

Table 4. Routine immunological lab assessment.

	Patient II:1		Patient II:3	
	Value	**Reference range**	**Value**	**Reference range**
White blood cells	*Age: 27 years*		*Age: 17 years*	
Total leukocytes (no./µL)	4860	3650-9300	5610	4500-12000
Neutrophils (no./µL)	2600	1573-6100	3350	2500-8000
Lymphocytes (no./µL)	1480	1133-3105	**1230**	1500-6500
$CD3^+$ T cells (no./µL)	1290	700-2100	886	700-2100
$CD3^+CD4^+$ T helper cells (no./µL)	636	300-1400	492	300-1400
$CD45RA^+$ naive CD4+ T cells (%)	**21**	33-66 †	**26**	33-66 †
$CD45RO^+$ memory CD4+ T cells (%)	**75**	18-38 †	**72**	18-38 †
$CD3^+CD8^+$ T cytotoxic cells (no./µL)	607	200-1200	283	200-1200
$CD45RA^+$ naive CD8+ T cells (%)	**47**	61-91 †	63	61-91 †
$CD45RO^+$ memory CD8+ T cells (%)	**51**	4-23 †	**35**	4-23 †
Ratio CD4/CD8	1.05	1.0-3.6	1.74	1.0-3.6
$CD19^+$ B cells (no./µL)	**89**	100-500	160	100-500
IgD^+CD27^- naive B cells (%)	63	48.4-79.7 ‡	**96**	48.4-79.7 ‡
$CD24^{++}CD38^{++}$ transitional B cells (%)	**27**	0.9-5.7 ‡	**23**	0.9-5.7 ‡
IgD^-CD27^+ switched memory B cells (%)	**28**	8.3-27.8 ‡	**0.5**	8.3-27.8 ‡
IgD^+CD27^+ marginal zone B cells (%)	**4**	7.0-23.8 ‡	**1**	7.0-23.8 ‡
$CD21^{low}$ $CD38^{low}$ B cells (%)	6	1.6-10.0 ‡	4	1.6-10.0 ‡
$CD3^-CD56^+CD16^+$ NK cells (no./µL)	104	90-600	172	90-600
Monocytes (no./µL)	420	247-757	900	500-1000
Eosinophils (no./µL)	**320**	28-273	110	100-500
Basophils (no./µL)	40	6-50	10	10-100
Immunoglobulins *	*Age: 17 years*		*Age: 13 years*	
IgG (g/L)	11.4	7.0-16.0	**5.7**	7.0-16.0
IgG2 (g/L)	1.57	1.06-6.1	1.94	1.06-6.1
IgG3 (g/L)	1.450	0.18-1.63	0.293	0.18-1.63
IgM (g/L)	**0.2**	0.40-2.48	**Undetectable**	0.34-2.14
IgA (g/L)	2.9	0.71-3.65	1.4	0.83-4.07
IgE (kU/L)	< 4.4	0-100	< 4.4	0-200
Specific antibody responses *	*Age: 17 years*		*Age: 10 years*	
S. pneumoniae polysaccharide IgG (Lab U)	**Undetectable**	≥ 11: immune	**4**	≥ 11: immune
Tetanus IgG (IU/mL)	0.2	≥ 0.01: immune	0.2	≥ 0.01: immune
Rubella IgG (IU/mL)	**< 8**	> 14: immune	NA	
Measles IgG (mIU/mL)	1400	> 300: immune	NA	
Mumps IgG (Lab U/mL)	**380**	> 500: immune	NA	
Varicella Zoster IgG (mIU/mL)	410	> 100: immune	NA	
Lymphocyte proliferation assay	*Age: 23 years*		*Age: 13 years*	
Response to Concanavalin A	Normal	Compared to control	Normal	Compared to control
Response to Phytohemagglutinin	Normal	Compared to control	Normal	Compared to control
Response to Tetanus toxoid	Normal	Compared to control	Normal	Compared to control
Response to Candida	Normal	Compared to control	Normal	Compared to control

The most recent, comprehensive and representative laboratory results are shown, with patient age at time of analysis mentioned. Aberrant values are indicated in bold. Both patients were immunized according to the recommended Belgian childhood immunization schedule that, among others, included tetanus, measles, mumps, and rubella vaccines. A polysaccharide (unconjugated) pneumococcal vaccine was given to both patients at time of immunological evaluation. The patients were not vaccinated against varicella zoster virus but had chickenpox in early childhood. *Measured when not receiving immunoglobulin replacement therapy. †Reference values from Shearer *et al.*[31] ‡Reference values from Piatosa *et al.*[32] NA: not available.

Despite elaborate diagnostic workup, the cause of the chronic enteropathy in patient II:3 could not be found. Severe failure to thrive (below the 1st percentile for height and weight) and poor intake eventually resulted in the need for total parental nutrition (TPN) from the age of 2 to 5 years. In that period, he also had total lymphopenia (Figure 3) and reduced T cell proliferative responses to stimulation with mitogens (PHA, Concanavalin A). At 9 months of age, he was hospitalized for herpes simplex encephalitis (positive herpes simplex virus type 1 (HSV-1) PCR testing on cerebrospinal fluid), which responded well to intravenous treatment with acyclovir. In his 3rd and 4th year of life, he had recurrent episodes of autoimmune anemia and two episodes of central venous catheter-related sepsis with enterococci and coagulase-negative staphylococci. After the age of 5 years, his clinical and laboratory phenotype remarkably ameliorated, with resolution of the chronic enteropathy, catch-up growth and cessation of TPN as well as a gradual increase of T cell numbers (Figure 3), normalization of mitogen-induced T cell proliferation, and normalization of serum IgA. Up to this day, however, he has mild total lymphopenia and absent serum IgM (Figure 3, Table 4). Similar to his sister (II:1), global antibody response to polysaccharide pneumococcal vaccine was impaired, total B cell numbers fluctuated around the lower limit of normal and transitional B cell levels were increased (Table 4, Figure 3). However, patient II:3 had very low switched memory B cells, which is the opposite of what was observed in his sister (II:1) (Table 4). When he was 9 years old, immunoglobulin therapy was interrupted for several months revealing decreased serum levels of total IgG. Analogously, in early adolescence, total IgG levels were reduced during a period of poor therapy compliance (Table 4). Together, this supported the diagnosis of CVID in patient II:3.[30] Today, he is doing clinically well under regular immunoglobulin replacement therapy and has reached his predicted height (12th percentile). Repeated imaging in patients II:1 and II:3 has, so far, not shown signs of lymphoproliferation, granulomata or bronchiectasis.

The father (I:1) was diagnosed with distal esophageal adenocarcinoma at the age of 45 years, secondary to chronic gastroesophageal reflux disease complicated by barrett's esophagus. He had two recurrences and deceased at 50 years of age. There were no clinical or laboratory signs of immunodeficiency in his past medical history. The mother (I:2) and third sibling (II:2) are in good health and basic immunological lab phenotyping was normal.

The affected siblings were compound heterozygous for novel *GTF3A* variants predicted to disrupt zinc finger formation and nucleic acid binding

To determine the underlying genetic defect, WES was performed in the two affected siblings (II:1, II:3) and both parents (I:1, I:2). Prior to WES, large pathogenic copy number variations (> 100 kb) were ruled out in both patients by routine array CGH. WES did not reveal good

candidate variants in known PID genes or other genes known to be involved in immunological responses. Interestingly, both affected siblings were compound heterozygous for two missense variants in *GTF3A* (NM_002097: c.585T>G, p.C195W and c.655T>C, p.C219R) predicted to be pathogenic and segregating with disease (Figure 2A, Table 5). Furthermore, both *GTF3A* variants were absent from public databases (dbSNP, ESP, GoNL, 1000 Genomes, ExAC, gnomAD) and from an in-house database containing variants from more than 1000 exomes at time of analysis. The *GTF3A* variants p.C195W and p.C219R were located in Cys_2His_2 zinc finger motifs 6 and 7, respectively (Figure 2B). Previous biochemical experiments demonstrated that zinc fingers 6 and 7 are involved in binding the *5S rDNA* promoter region and the *5S rRNA* transcript.[10] For both *GTF3A* variants, one of the two highly conserved, negatively charged, cysteine residues essential for zinc finger formation was replaced by a bulky, non-negatively charged, amino acid (Figure 2B). These amino acid substitutions were, therefore, likely to disrupt zinc ion binding, destabilizing the 3D zinc finger structure necessary for nucleic acid binding (Figure 2B). Notwithstanding that *GTF3A* is ubiquitously expressed in human tissues, publically available mRNA expression datasets showed that it is highly expressed in all lymphocytes and highest in B cells (http://biogps.org/). Altogether, this made *GTF3A* a strong candidate gene for the immunodeficiency phenotype observed in the here-presented patients.

Table 5. *In silico* pathogenicity predictions of the *GTF3A* variants.

	c.585T>G, p.C195W	**c.655T>C, p.C219R**
Conservation nucleotide	Weak	High
Conservation amino acid	High, up to Fruitfly (considering 14 species)	High, up to Fruitfly (considering 14 species)
Grantham distance	215	180
Align GVGD	C15	C0
SIFT	Deleterious	Deleterious
PolyPhen-2	Probably Damaging	Probably Damaging
CADD phred score (MSC: 4.921)	27.2	27.8

By Alamut Visual software, **nucleotide conservation** is determined based on phastCons and phyloP scores,[33,34] and **amino acid conservation** through orthologue alignments and information on protein domains. **Grantham distance** reflects the differences in physicochemical properties between amino acids. Grantham scores range from 5 to 215; higher scores are considered more deleterious with values ≥ 150 considered radical.[35] **Align-GVGD** combines the biophysical characteristics of amino acids and protein multiple sequence alignments to predict pathogenicity of missense variants. Align-GVGD predictions are shown as classes (C0 to C65) with class ≥ C45 considered to interfere with function.[36] **SIFT** (Sorting Intolerant From Tolerant) predicts the possible impact of amino acid substitutions based on sequence homology and the physical properties of amino acids.[37] **PolyPhen-2** (Polymorphism Phenotyping v2) predicts the possible impact of missense variants on the stability and function of human proteins using structural and comparative evolutionary considerations.[38] **CADD** (Combined Annotation Dependent Depletion) scores the deleteriousness of single nucleotide variants as well as insertion/deletion variants in the human genome.[39] CADD phred scores ≥ 20 predict that a variant is among the 1% most deleterious of all possible substitutions. **MSC** (Mutation Significance Cutoff) is a quantitative approach that provides gene-specific phenotypic impact cut-off values for the CADD score.[40]

TFIIIA expression is normal whereas *5S rRNA* expression is reduced in the patients

TFIIIA protein expression was comparable between the two affected siblings, unaffected sister, mother and healthy controls (Figure 4A). In contrast, transcript expression levels of *5S rRNA*, the main target of TFIIIA, were significantly lower in the patients compared to the mother and healthy controls (Figure 4B). $tRNA^{Leu}$ and $tRNA^{Tyr}$, two TFIIIA-independent genes, were similarly expressed in all studied subjects (Figure 4B). These expression data support the hypothesis of loss of TFIIIA function in the patients, while TFIIIA-independent Pol III transcriptional activity appears to be intact.

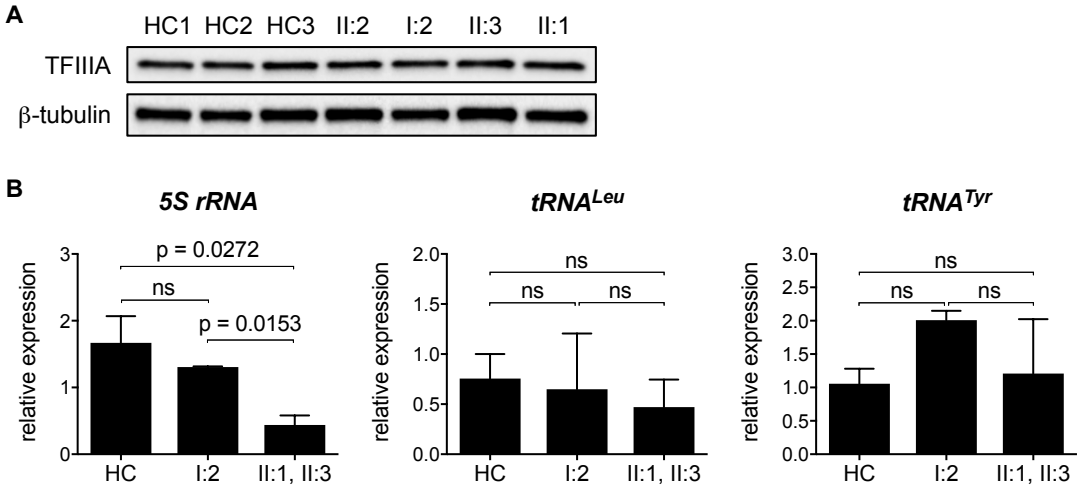


Figure 4. Expression of TFIIIA, *5S rRNA*, $tRNA^{Leu}$ and $tRNA^{Tyr}$. (A) TFIIIA protein expression evaluated by western blot. β-tubulin was used as loading control. **(B)** Transcript expression levels of the TFIIIA-dependent gene *5S rRNA* and the TFIIIA-independent genes $tRNA^{Leu}$ and $tRNA^{Tyr}$, determined by qPCR. Data represent mean ± SEM of two replicate experiments (2-3 HC per experiment). The three groups were compared by use of one-way ANOVA. If this indicated significant differences, 2-by-2 comparisons were done with the unpaired Student's t test. ns: not significant. All analyses were performed on unstimulated total PBMCs of the affected siblings (II:1, II:3), the unaffected mother (I:2) and healthy controls (HC). Since material of the unaffected sister (II:2) was limited, only TFIIIA protein expression could be determined.

Patients' B cells demonstrated intact surface IgM expression, normal activation and cytokine secretion, but altered differentiation *in vitro*

A possible role of TFIIIA in the immune system is thus far unknown. To assess which pathways might be affected by mutant TFIIIA, we performed detailed immunophenotypic analysis of PBMCs (as previously described[26]) and general immuno-functional screening of B cells, T cells and TLR signaling.

Despite the fact that serum IgM is reduced to undetectable in the patients, surface IgM expression on B cells was comparable to that of healthy controls (Figure 5A). Moreover, upon stimulation of patients' PBMCs with anti-IgM, phosphorylation of the immediately downstream effector proteins BTK and PLCγ2 was normal (Figure 5B). Hence, surface IgM in the patients was not only present but also capable of signal transduction.

To evaluate the overall B cell activation response, patients' PBMCs were treated with different B cell stimuli for 4 days. After anti-IgM/CD40L stimulation, upregulation of the B cell activation markers CD80, CD25 and CD95 was normal in the patients compared to the mother and healthy controls (Figure 5C). Similar results were observed after IgM-independent stimulation with IL-21/CD40L or CpG/CD40L (data not shown). Note that anti-IgM will mainly activate naive B cells and IgM^+ memory B cells, whereas IgM-independent stimuli will mostly trigger memory B cells.[41,42] In correspondence with the adequate B cell activation responses, patients' B cells secreted normal amounts of cytokines upon stimulation with anti-IgM/CD40L for 4 days (Figure 5D).

In vitro differentiation of B cells into $CD38^{high}CD27^{high}$ plasmablasts was examined by stimulating patients' PBMCs with IL-21/CD40L or CpG/CD40L for 4 days (Figure 5E).[43] Stimulation with anti-IgM/CD40L was used as negative control (Figure 5E, top graphs).[44] In patient II:3, plasmablast formation was reduced after IL-21/CD40L and virtually absent after CpG/CD40L stimulation (Figure 5E). In patient II:1, however, plasmablast generation was normal after CpG/CD40L and markedly elevated after IL-21/CD40L compared to healthy controls (Figure 5E). These findings corresponded with the patients' *in vivo* levels of switched memory B cells (Table 4).

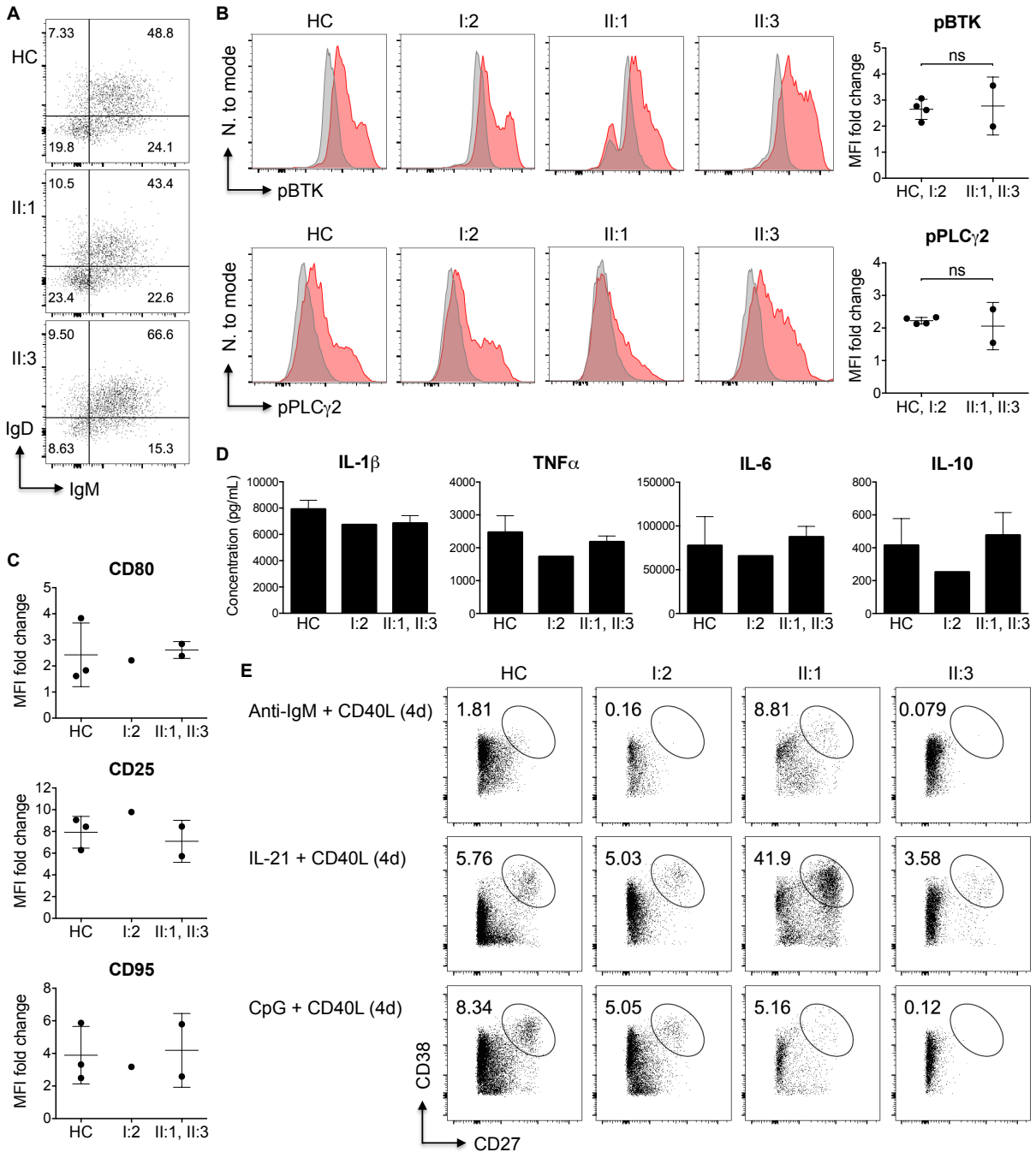


Figure 5. IgM surface expression, and B cell activation, cytokine secretion and differentiation. **(A)** IgM surface expression on resting CD19^{+} B cells of the patients (II:1, II:3) compared to an adult healthy control (HC). **(B)** Total PBMCs were stimulated with anti-IgM for 10 min. BTK and PLCγ2 phosphorylation, evaluated in CD19^{+} B cells, was similar in the patients compared to the mother (I:2) and HC (unpaired Student's t test). Graphs show mean ± SD. ns: not significant, MFI: mean fluorescence intensity. **(C)** Total PBMCs were stimulated with anti-IgM and CD40L for 4 days. Upregulation of the activation markers CD80, CD25 and CD95 was determined on CD19^{+} B cells. Graphs show mean ± SD. MFI: mean fluorescence intensity. **(D)** Total PBMCs were stimulated with anti-IgM and CD40L for 4 days, and cytokine secretion was measured in harvested supernatant of the patients, mother and 3 HC. Graphs show mean ± SD. **(E)** Total PBMCs were stimulated as indicated. After 4 days (4d), *in vitro* differentiation of B cells into CD27^{high}CD38^{high} plasmablasts was evaluated. Representative data of two (A), three (B, C) or one (D, E) replicate experiment(s) are displayed. HC: healthy control.

B cell proliferation responses were impaired in both patients

B cell proliferation upon *in vitro* stimulation was significantly reduced in the patients compared to the unaffected mother and healthy control subjects (Figure 6). This was seen for all tested combinations of B cell stimuli. In patient II:1, percentages of divided B cells in the IL-21 condition were normal, but there was still a large undivided peak (i.e. utmost right peak on graph) that was not seen in the healthy controls nor the mother (Figure 6A). In accordance, proliferation indices (representing the number of divisions a cell has undergone) were significantly lower in the patients compared to the mother and healthy controls for all tested conditions (Figure 6B). The B cell proliferation defect seemed to be most pronounced in the patients' naive B cell compartment (Figure 6C). Particularly after stimulation with IL-21, the patients' naive B cells showed a large undivided peak while naive B cells of the mother and healthy controls all went into division (Figure 6C).

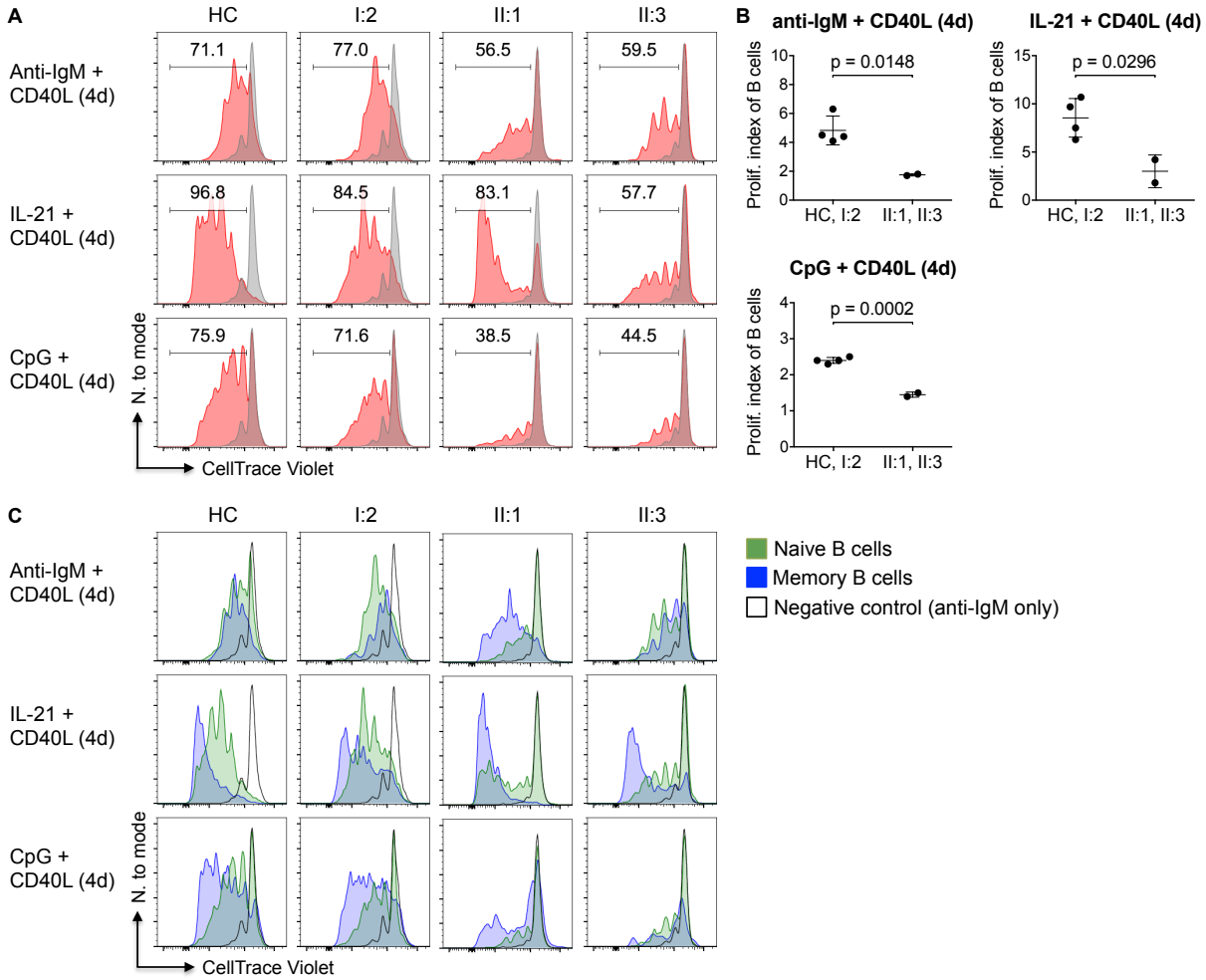


Figure 6. Impaired B cell proliferation. Total PBMCs were stimulated as indicated for 4 days (4d). Data in all panels are derived from the same samples and are representative for three replicate experiments. **(A)** Proliferation of total $CD19^+$ B cells measured as the percentage of divided cells based on dilution of the CellTrace Violet proliferation dye. Histograms depict stimulated cells (red shaded) versus anti-IgM only treated cells (grey shaded, negative control). **(B)** Proliferation (Prolif.) indices of the same conditions presented in panel (A). Graphs show mean ± SD. Statistical significance was determined using the unpaired Student's t test. **(C)** Proliferation of naive ($CD27^-$) and memory ($CD27^+$) B cells, derived from the same samples presented in panel (A). Total B cells only treated with anti-IgM were used as negative control. HC: healthy control.

T cell receptor induced proliferation is impaired while overall signaling appears unaltered

Detailed immunophenotyping of peripheral T cell subsets did not reveal any abnormalities in the patients compared to age-matched healthy controls (data not shown).

Upon stimulation with anti-CD3/anti-CD28 Dynabeads, the T cell proliferation histograms showed a large undivided peak in the patients that was absent in the mother and healthy controls (Figure 7A, top). Indeed, the corresponding proliferation indices were significantly reduced in the patients (Figure 7B, top). However, T cell proliferation after stimulation with the mitogen PHA was normal in both patients (Figure 7A-B, bottom). Proliferation differences between naive and memory T cells were not yet evaluated.

Analogous to the normal activation and cytokine production by patients' B cells, upregulation of the T cell activation marker CD25 at day 4 as well as T cell cytokine secretion at 24 hours after stimulation was similar in the patients, mother and healthy controls (Figure 7C-D).

To evaluate downstream T cell receptor signaling, western blot was performed for the proximal effector molecule PLCγ1, several key components of the NFκB pathway, and ERK as overall measure of the MAPK signaling pathway (Figure 7E). Both total levels and phosphorylation of the investigated proteins were comparable between patients, their mother and healthy controls (Figure 7E).

Finally, STAT1 and STAT5 phosphorylation in $CD4^+$ T cells upon cytokine stimulation were also normal (Figure 7F).

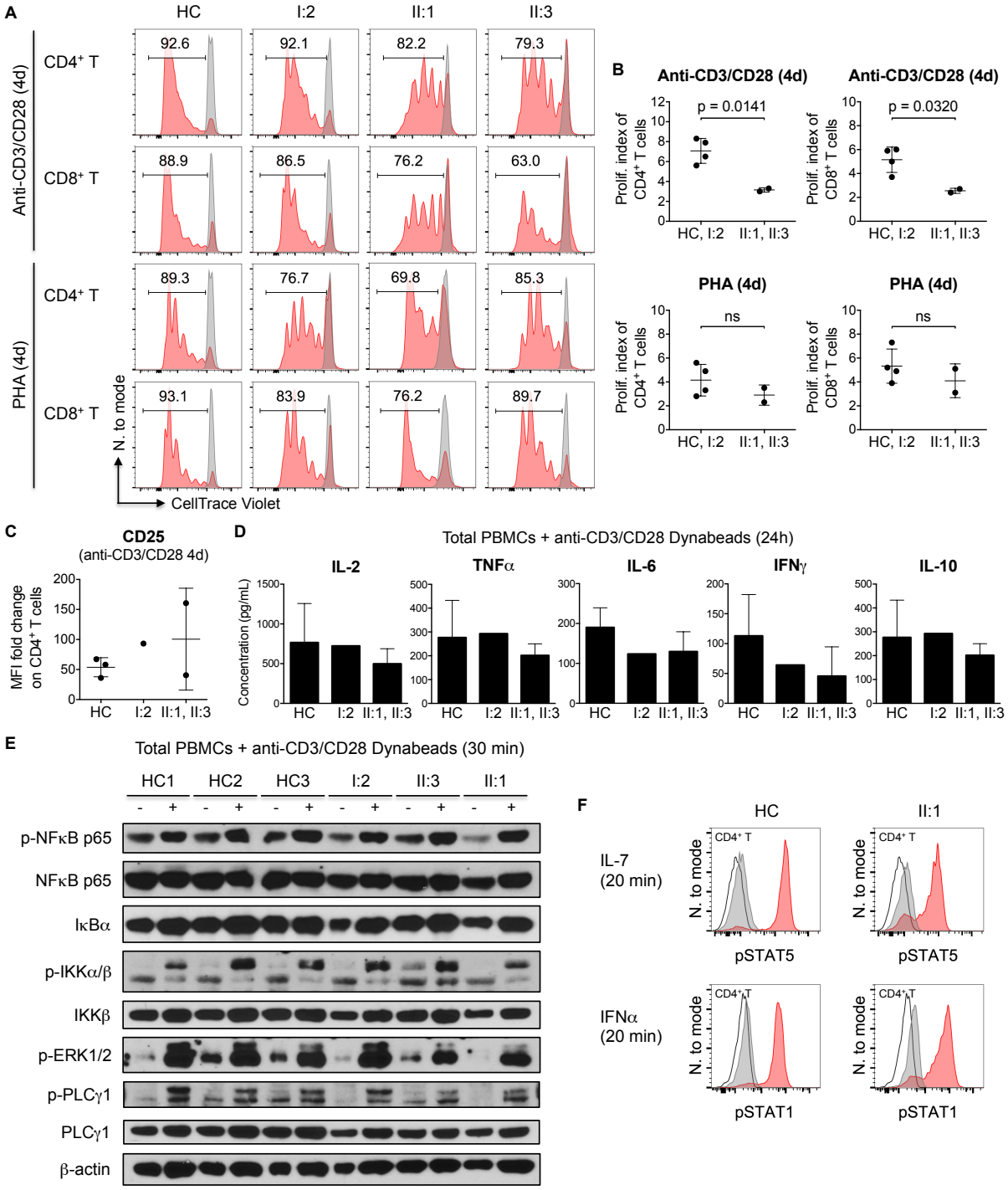


Figure 7. T cell proliferation, activation, cytokine secretion and signaling. (A) Total PBMCs were stimulated as indicated for 4 days (4d). Proliferation of $CD4^+$ and $CD8^+$ T cells was measured as the percentage of divided cells based on dilution of the CellTrace Violet proliferation dye. Histograms depict stimulated (red shaded) versus unstimulated cells (grey shaded). PHA: phytohaemagglutinin. **(B)** Proliferation (Prolif.) indices of the same conditions presented in panel (A). Graphs show mean ± SD. Statistical significance was determined using the unpaired Student's t test. ns: not significant. **(C)** Upregulation of the activation marker CD25 on $CD4^+$ T cells after 4 days (4d) of stimulation with anti-CD3/CD28 Dynabeads. Graph shows mean ± SD. MFI: mean fluorescence intensity. **(D)** Total PBMCs were stimulated as indicated, and cytokine secretion was measured in the patients, mother and 3 HC. Graphs show mean ± SD. **(E)** Total PBMCs were stimulated with anti-CD3/CD28 Dynabeads for 30 min (+) or left untreated (-). T cell receptor signaling molecules were evaluated by western blot. **(F)** STAT1 and STAT5 phosphorylation in $CD4^+$ T cells after cytokine stimulation of total PBMCs. Histograms depict unstimulated cells (grey shaded), stimulated cells (red shaded), and IgG isotype control (black line). Representative data of two (A, B, C), three (D, E) or one (F) replicate experiment(s) are shown. HC: healthy control.

Limited screening of the innate immune system in the patients was normal

As part of the first-step general functional screening pipeline, limited testing of the innate immune system was performed. The patients did not show alterations in peripheral NK cell, monocyte or dendritic cell subsets (data not shown). Furthermore, cytokine secretion after stimulation with the TLR4-agonist LPS was comparable to that of healthy controls (Figure 8). Analogous to the normal STAT1 phosphorylation in $CD4^+$ T cells (Figure 7F), IFNγ-induced STAT1 phosphorylation in monocytes was also normal (data not shown).

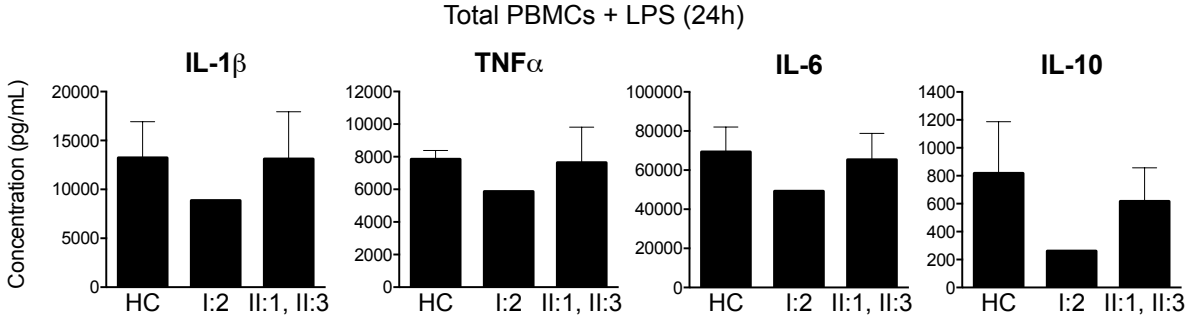


Figure 8. LPS-induced cytokine secretion. Total PBMCs of the patients, mother and 3 healthy controls (HC) were stimulated with the TLR4-agonist LPS for 24 hours (24h). Cytokine secretion was measured in harvested supernatant. Graphs show mean ± SD. Data is representative of three replicate experiments.

Mutant TFIIIA did not affect NFκB signaling activity in HEK293T cells

It was recently described that canonical NFκB signaling is broadly impaired among CVID patients with reduced switched memory B cells, independent of the clinical phenotype or genetic defect.[45] In the here-reported patients, patient II:3 had very low switched memory B cells whereas patient II:1 had high switched memory B cells. To determine the specific effect of the mutant *GTF3A* alleles on NFκB signaling, independent of the patients' genetic background, an NFκB luciferase reporter assay was performed (Figure 9). HEK293T cells were transfected with WT, one mutant or both mutant *GTF3A* plasmids, along with a luciferase reporter gene construct for NFκB. Luciferase signal was measured after 6 hours stimulation with TNFα. NFκB signaling activity induced by the TFIIIA mutants, either separately or combined, was comparable to that of WT TFIIIA (Figure 9). These findings are in line with the normal NFκB signaling after TCR stimulation (Figure 7E).

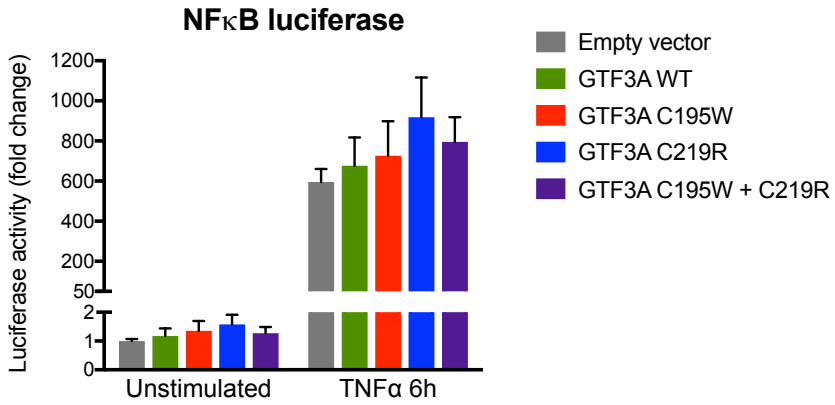


Figure 9. NFκB luciferase reporter assay. HEK293T cells were transfected with *GTF3A* wild type (WT) or mutant (single or combined) plasmids along with the NFκB reporter construct. Transfection with empty vector was used as control and for normalization. Twenty-four hours after transfection, cells were stimulated with TNFα for an additional 6 hours (6h) or left untreated. Reporter luciferase activity was subsequently measured. There were no significant differences in luciferase activity between cells transfected with empty vector, WT *GTF3A*, single mutant *GTF3A* or combined mutant *GTF3A* (one-way ANOVA). Data represent mean ± SEM of five independent experiments.

Normal intracellular localization of mutant TFIIIA

We found that *5S rRNA* expression was significantly reduced in patients' PBMCs (Figure 4), suggestive of reduced transcriptional activity of TFIIIA. Therefore, the effect of the *GTF3A* variants on nuclear translocation of the protein was investigated. NIH-3T3 cells were transfected with WT, single mutant or combined mutant *GTF3A* vectors and TFIIIA was detected by fluorescently labeled antibody. Fluorescence microscopy showed no difference in intracellular localization patterns between WT and mutants, implicating that the two *GTF3A* missense variants did not disrupt nuclear translocation of TFIIIA (data not shown).

Transient TFIIIA depletion mildly reduced *5S rRNA* expression and B cell proliferation

To examine whether a reduction of TFIIIA function was responsible for the diminished *5S rRNA* expression in the patients, we knocked down *GTF3A* in healthy donor PBMCs by use of siRNA. Transfection efficiency was rather low (~25%) but consistent between experiments. qPCR at 48 hours posttransfection showed a mild decrease in *5S rRNA* expression in TFIIIA-depleted cells, but this did not reach statistical significance ($p = 0.058$) (Figure 10A). Expression of the control targets $tRNA^{Leu}$ and $tRNA^{Tyr}$ at 48 hours posttransfection was very variable across experiments, but was on average unchanged (Figure 10A). Although transient *GTF3A* knockdown with siRNA is expected to wear off after a few days, in 4 out of 6 experiments *GTF3A* expression was still silenced at day 5 to 6 posttransfection. At that time, *5S rRNA* expression was found to be significantly reduced in TFIIIA-depleted cells (Figure 10B). To assess whether transient TFIIIA depletion had an

observable effect on B and T cell proliferation, transfected cells were dyed with CellTrace Violet and stimulated with anti-IgM/CD40L or anti-CD3/CD28 Dynabeads, respectively, for 4 to 5 days starting 24 hours posttransfection. B cell proliferation of TFIIIA-depleted cells was mildly reduced compared to that of cells transfected with control siRNA, although this did not reach statistical significance (p = 0.0536) (Figure 10C). In contrast, proliferation of $CD4^+$ and $CD8^+$ T cells was not altered in TFIIIA-depleted cells (data not shown).

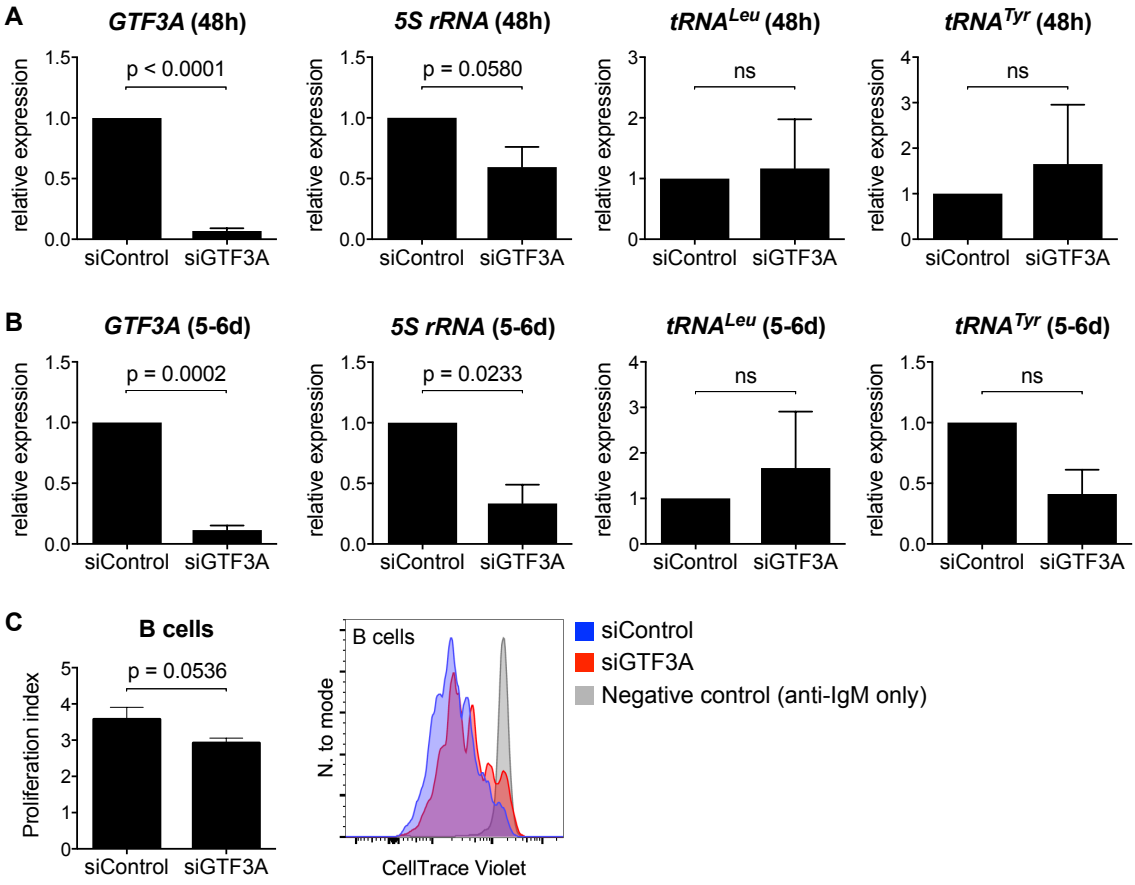


Figure 10. Transient *GTF3A* knockdown in healthy donor PBMCs. Cells were transfected with siRNA-universal negative control (siControl) or siRNA-GTF3A (siGTF3A). **(A)** Relative gene expression at 48 hours posttransfection, evaluated with qPCR. **(B)** Relative gene expression at 5-6 days posttransfection, evaluated with qPCR. **(C)** To examine B cell proliferation, cells were stained with CellTrace Violet proliferation dye and stimulated with anti-IgM/CD40L for 4 to 5 days starting 24 hours posttransfection. A representative histogram is depicted on the right. Data from six (A) or four (B, C) independent experiments are shown. All graphs represent mean ± SEM. Statistical significance was determined using the paired Student's t-test. ns: not significant.

DISCUSSION

We identified novel compound heterozygous variants in *GTF3A*, encoding the transcription factor TFIIIA,[4] in a Belgian brother and sister pair presenting with a CVID-like phenotype. Thus far, *GTF3A* has not been associated with human disease and a possible role of TFIIIA in the immune system remains enigmatic. Computational predictions as well preliminary functional studies have provided evidence that the here-reported *GTF3A* variants could be disease-causing through loss of TFIIIA function. In particular, the variants were located in highly conserved cysteine residues of zinc finger motifs 6 and 7, respectively, and were predicted to destabilize the corresponding 3D zinc finger structures and subsequent nucleic acid binding. TFIIIA protein expression in the patients was similar to that in the unaffected family members and healthy controls. Expression of *5S rRNA*, the best-established TFIIIA target gene,[6,7] was significantly reduced in the patients compared to the heterozygous mother and healthy controls, whereas expression of TFIIIA-independent tRNA-coding genes appeared to be unaltered.

Based on the normal TFIIIA expression, the reduced but not completely absent *5S rRNA* expression, and the fact that each variant only disrupts one of nine consecutive zinc fingers of TFIIIA, we hypothesize that the patients have two partial loss-of-function *GTF3A* alleles resulting in two aberrant TFIIIA proteins that each lost part of their nucleic acid binding capacity. Since *5S rRNA* is important in ribosome biogenesis,[6-8] the two hypomorphic *GTF3A* alleles may result in a novel ribosomopathy associated with primary antibody failure.[17,20]

Both siblings, currently 28 (II:1) and 18 (II:3) years old, had recurrent (mainly respiratory tract) infections since early childhood, reduced to absent serum IgM, impaired antibody responses to polysaccharide pneumococcal vaccine, and low-normal total B cells. The boy (II:3) also demonstrated decreased levels of total IgG and mild lymphopenia. Consistent with the laboratory phenotype, patient II:3 had a more severe clinical course than his older sister. In early childhood, the boy suffered from chronic idiopathic enteropathy resulting in severe failure to thrive and need for TPN for several years. During that time he showed evidence of a combined immunodeficiency with T cell lymphopenia and panhypogammaglobulinemia. However, once the gastrointestinal symptoms resolved, his immunological lab phenotype ameliorated into a CVID-like picture. This suggests that the more severe immunosuppression in the first years of life might have been secondary to the enteropathy and/or the underlying cause of the enteropathy. We presume that the chronic intestinal inflammation in patient II:3 was most likely triggered by an infectious agent. In support of this, there have been sporadic reports on immunodeficient patients with persistent enteric (viral) infections that subsequently developed inflammatory enteropathy.[46] Furthermore, viral infections are notorious for

inducing transient T cell lymphopenia[47], although the prolonged course and the residual persistent mild lymphopenia seen in patient II:3 were probably associated with his primary immunodeficiency.

Preliminary functional studies on patients' PBMCs uncovered an important B cell proliferation defect, irrespective of stimulation through the B cell receptor or through cytokine receptors. Remarkably, total B cell proliferation percentages were normal in patient II:1 after stimulation with IL-21, although there was still a large peak of undivided cells. It was previously shown that memory B cells possess an intrinsic advantage over naive B cells in terms of proliferation and differentiation after *in vitro* stimulation.[48] Indeed, subsequent investigations revealed that especially the naive B cells in both patients proliferated very poorly while memory B cell proliferation was close to normal upon IL-21 stimulation. Since patient II:1 had > 25% memory B cells and patient II:3 had < 1%, this probably explains why total B cell proliferation percentages after stimulation with IL-21 were markedly higher in patient II:1 than in patient II:3. Still, it is unclear why the patients' (memory) B cells proliferated more after IL-21/CD40L stimulation than after anti-IgM/CD40L or CpG/CD40L stimulations.

Besides impaired B cell proliferation, less-pronounced defects in T cell proliferation were detected. Remarkably, T cell proliferation was significantly reduced after anti-CD3/CD28 but not after PHA stimulation. Anti-CD3/CD28 specifically triggers the T cell receptor whereas PHA engages surface glycoproteins in an aspecific manner.[49] As such, PHA stimulates IL-6 production by monocytes, which, in turn, enhances T cell proliferation.[50] It is possible that, in contrast to the B cell situation, adding cytokines to anti-CD3/CD28 could overcome the T cell proliferation defect. The latter needs to be further explored. Moreover, differences between naive and memory T cell proliferation will also be investigated in the near future.

In contrast to the defects in B and T cell proliferation, both patients showed normal upregulation of B and T cell surface activation markers, normal B and T cell cytokine production, normal proximal B and T cell receptor signaling, normal T cell receptor-induced NFκB and ERK signaling, normal cytokine-induced STAT1 and STAT5 phosphorylation in T cells, and normal LPS-induced cytokine production. Furthermore, the *GTF3A* variants did not affect NFκB reporter luciferase activity in HEK293T cells. Altogether, these data suggested that, despite the proliferation defect, overall activation and signaling machineries in the patients' immune cells appeared to be intact. In fact, since proliferation responses were impaired upon stimulation through the B cell receptor, B cell cytokine receptors, as well as the T cell receptor, a defect in a common cell cycle-related mechanism is suspected.

OUTSTANDING QUESTIONS AND STRATEGIES TO ADDRESS THEM

5S rRNA (the main target of TFIIIA) is a component of the 60S large subunit of ribosomes.[8] Ribosome synthesis and 60S subunit maturation have been previously studied in yeast models for Diamond-Blackfan anemia and Shwachman-Diamond syndrome, two well-known ribosomopathies.[51] To examine the effect of the *GTF3A* variants on **60S subunit synthesis**, we will first perform **polysome profiling** in patient cells, which visualizes the presence of the different ribosome subunits in the form of a peak diagram.[51] Since *5S rRNA* expression was found to be decreased in the patients, we expect a similar reduction in 60S subunit synthesis. Afterwards, a direct link between TFIIIA and reduced 60S synthesis could be further investigated using a relevant cellular model (see below, CRISPR/Cas9 genome editing technique).

The lymphocyte proliferation defect observed in the *GTF3A*-mutant patients was particularly interesting because ribosome biogenesis and 5S rRNA play key roles in the regulation of cell cycle progression.[8,17,18,52] When ribosome biogenesis is impaired, 5S rRNA is redirected from assembly into nascent 60S ribosomes and becomes an essential element in the signaling cascade inducing p53-dependent cell cycle arrest.[8,52] In the absence of 5S rRNA, p53-independent cell cycle arrest can be induced.[8,52] Moreover, defects in cell cycle progression as well as variable B and T cell proliferation defects have been previously documented in several ribosomopathies.[17,20] The role of TFIIIA and 5S rRNA in lymphocyte cell cycle is unknown. We, therefore, plan to do **flow cytometric cell cycle analysis** on patients' PBMCs using a DNA-binding dye and counterstaining for (naive and memory) B and T cell markers. PBMCs will be stimulated with anti-IgM/CD40L or anti-CD3/anti-CD28 to stimulate B or T cell proliferation, respectively. Furthermore, defects in SBDS, the underlying cause of Shwachman-Diamond syndrome, have been implicated to destabilize the mitotic spindle (a microtubule-based structure that separates chromosomes during mitosis) in human hematopoietic progenitor cells and lymphoblasts.[53,54] Hence, we will investigate if TFIIIA colocalizes with the **mitotic spindle** in healthy donor cells (e.g. PHA lymphoblasts, skin fibroblasts) by use of immunofluorescence.[53,54] If so, the effect of the mutant TFIIIA proteins on mitotic spindle formation and cell division will be further explored.[54]

The rate of cell growth and cell proliferation is largely determined by the rate of protein synthesis by ribosomes.[8] Most species, including humans, have multiple gene copies of *5S rDNA*.[9] Studies in *Escherichia coli (E. coli)* have shown that multiple *5S rDNA* gene copies were not necessary for survival but allowed the bacteria to rapidly upregulate 5S rRNA increasing their growth potential.[55] In particular, deletion of 75% of the *5S rDNA* gene copies in an *E. coli* strain was not lethal to the bacteria but markedly reduced growth (proliferation)

rates.[55] In human cancer cell lines, siRNA-mediated *GTF3A* knockdown did not reduce total *5S rRNA* expression levels up to 72 hours posttransfection.[8,56] Correspondingly, a decrease in cell proliferation only became visible after 72 to 96 hours posttransfection.[56] Analogously, in our experiments, transient *GTF3A* knockdown in healthy donor PBMCs did not significantly reduce *5S rRNA* expression after 48 hours, but did after 5 to 6 days posttransfection. Still, after 5-6 days, proliferation of TFIIIA-depleted B cells was not significantly reduced although it showed a decreasing trend. T cell proliferation was normal in TFIIIA-depleted cells. A reason as to why we were unable to detect a proliferation defect after siRNA-mediated knockdown of *GTF3A*, may be because PBMCs have a lower, less demanding, proliferation rate compared to human cancer cell lines and *E. coli* bacteria.[55-57] Indeed, lower resource needs in PBMCs could make it more difficult to pick up a proliferation defect in a transient transfection setting, particularly because 5S rRNA is not yet sufficiently depleted in the first 48-72 hours posttransfection. In the future, we wish to generate a **mutant *GTF3A* knock-in model** in primary healthy control B and T cells using the **CRISPR/Cas9** genome editing technology.[58,59] This technology allows stable DNA sequence alterations in eukaryotic cells, generating a more suitable cellular system to investigate the effect of mutant TFIIIA on both **B and T cell proliferation** and **ribosome synthesis** (see above) independent of the patients' genetic background.

Next to variable B and T cell proliferation defects, several ribosomopathies have been associated with various degrees of B cell and/or T cell lymphopenia, reduced serum IgM, IgA, IgG and/or IgG subclasses, and/or impaired specific antibody responses to immunization.[20] Furthermore, there have been reports on patients with well-established ribosomopathies that were diagnosed with CVID.[20] Importantly, the immunodeficiency phenotype of the here-reported patients bears striking similarities with the immunological abnormalities seen in ribosomopathies.[20] Patient II:1 had reduced IgM, whereas patient II:3 had absent IgM and reduced IgG serum levels. In addition, both patients had very poor antibody responses to polysaccharide pneumococcal vaccines. IgM deficiency with normal levels of IgA, IgG and IgG subclasses, as seen in the oldest sibling II:1, is rare and poorly understood.[60,61] Similar as in the here-presented patients, the majority of cases with IgM deficiency have intact surface IgM expression on B cells.[60,61] Furthermore, impaired polysaccharide antibody responses are often encountered in patients with IgM deficiency.[60] Total B cell numbers fluctuated around the lower limit of normal in both patients. Peculiarly, patient II:3 had < 1% switched memory B cells whereas sibling II:1 had very high (> 25%) switched memory B cells. This difference in B cell differentiation was also observed *in vitro*, where patient II:3 had reduced and patient II:1 had normal to elevated plasmablast formation. The dissimilarity in B cell differentiation between the two siblings is not easily explained.

Overall, patient II:3 had a more severe clinical and laboratory phenotype than his sister (II:1). Over the past years, it has become clear that most PID- or ribosomopathy-related genes, including recessive ones, are associated with considerable phenotypical variability even within the same family.[3,17,62] It is possible that the dissimilarity in switched memory B cells in particular, and disease severity in general, is part of a phenotypical spectrum. Additionally, it cannot be excluded that natural disease progression has been influenced by environmental factors such as exposure to infectious pathogens or administration of immunoglobulin replacement therapy. Indeed, immunoglobulin therapy has been shown to inhibit B cell differentiation *in vitro*.[63] Besides a few temporary interruptions, patient II:3 has been on immunoglobulin replacement therapy since he was 7 months of age. In contrast, patient II:1 started immunoglobulin therapy when she was 18 years old. Unfortunately, studies on long-term *in vivo* effects of immunoglobulin replacement therapy on peripheral B cell differentiation are lacking.

To date, the full transcriptome of the transcription factor TFIIIA is unknown. To address the abnormalities in antibody production and memory B cell differentiation, we plan to do **transcriptome analysis** using a combination of two techniques: Chromatin Immuno-Precipitation followed by next-generation sequencing (**ChIP-seq**) and RNA-sequencing (**RNA-seq**). ChIP-seq will reveal the DNA binding sites of TFIIIA and whether these are affected by the *GTF3A* variants, while RNA-seq will show possible consequences on gene expression. Since the patients' defects appear to be quite cell type specific, ChIP-seq and RNA-seq will be performed on purified B cells of the patients (II:1, II:3) in comparison to healthy controls as well as to the heterozygous mother (I:2) and WT/WT sister (II:2) as controls for genetic background. ChIP-seq and RNA-seq data can then be integrated to evaluate the consequences of the *GTF3A* variants on transcription regulation. We believe that transcriptional profiling may provide additional clues towards the patients' phenotypes.

CONCLUSION

We have identified compound heterozygous *GTF3A* variants as potential cause of a novel ribosomopathy featured by a CVID-like phenotype; thereby revealing a previously unknown role of TFIIIA in lymphocyte proliferation and B cell biology. Future studies are planned to investigate the causal relationship between the *GTF3A* genotype and the clinical phenotype via relevant cellular models.

References

1. Bonilla FA, Barlan I, Chapel H, et al. International Consensus Document (ICON): Common Variable Immunodeficiency Disorders. J Allergy Clin Immunol Pract. 2016;4(1):38-59.
2. Modell V, Quinn J, Orange J, Notarangelo LD, Modell F. Primary immunodeficiencies worldwide: an updated overview from the Jeffrey Modell Centers Global Network. Immunol Res. 2016;64(3):736-53.
3. Bogaert DJ, Dullaers M, Lambrecht BN, Vermaelen KY, De Baere E, Haerynck F. Genes associated with common variable immunodeficiency: one diagnosis to rule them all? J Med Genet. 2016;53(9):575-90.
4. Moorefield B, Roeder RG. Purification and characterization of human transcription factor IIIA. J Biol Chem. 1994;269(33):20857-65.
5. Hanas JS, Hocker JR, Cheng YG, et al. cDNA cloning, DNA binding, and evolution of mammalian transcription factor IIIA. Gene. 2002;282(1-2):43-52.
6. Engelke DR, Ng SY, Shastry BS, Roeder RG. Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. Cell. 1980;19(3):717-28.
7. Oler AJ, Alla RK, Roberts DN, et al. Human RNA polymerase III transcriptomes and relationships to Pol II promoter chromatin and enhancer-binding factors. Nat Struct Mol Biol. 2010;17(5):620-8.
8. Donati G, Peddigari S, Mercer CA, Thomas G. 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. Cell Rep. 2013;4(1):87-98.
9. Sorensen PD, Frederiksen S. Characterization of human 5S rRNA genes. Nucleic Acids Res. 1991;19(15):4147-51.
10. Tan C, Li W, Wang W. Localized frustration and binding-induced conformational change in recognition of 5S RNA by TFIIIA zinc finger. J Phys Chem B. 2013;117(50):15917-25.
11. Pelham HR, Brown DD. A specific transcription factor that can bind either the 5S RNA gene or 5S RNA. Proc Natl Acad Sci U S A. 1980;77(7):4170-4.
12. Howe L, Ausio J. Nucleosome translational position, not histone acetylation, determines TFIIIA binding to nucleosomal Xenopus laevis 5S rRNA genes. Mol Cell Biol. 1998;18(3):1156-62.
13. Mathieu O, Yukawa Y, Prieto JL, Vaillant I, Sugiura M, Tourmente S. Identification and characterization of transcription factor IIIA and ribosomal protein L5 from Arabidopsis thaliana. Nucleic Acids Res. 2003;31(9):2424-33.
14. Layat E, Cotterell S, Vaillant I, Yukawa Y, Tutois S, Tourmente S. Transcript levels, alternative splicing and proteolytic cleavage of TFIIIA control 5S rRNA accumulation during Arabidopsis thaliana development. Plant J. 2012;71(1):35-44.
15. Woychik NA, Young RA. Genes encoding transcription factor IIIA and the RNA polymerase common subunit RPB6 are divergently transcribed in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. 1992;89(9):3999-4003.
16. Camier S, Dechampesme AM, Sentenac A. The only essential function of TFIIIA in yeast is the transcription of 5S rRNA genes. Proc Natl Acad Sci U S A. 1995;92(20):9338-42.
17. Yelick PC, Trainor PA. Ribosomopathies: Global process, tissue specific defects. Rare Dis. 2015;3(1):e1025185.
18. Farley KI, Baserga SJ. Probing the mechanisms underlying human diseases in making ribosomes. Biochem Soc Trans. 2016;44(4):1035-44.
19. Liu JM, Lipton JM, Ellis SR. Genetics of the Ribosomopathies. In: eLS. John Wiley & Sons Ltd, Chichester. Feb 2013.
20. Khan S, Pereira J, Darbyshire PJ, et al. Do ribosomopathies explain some cases of common variable immunodeficiency? Clin Exp Immunol. 2011;163(1):96-103.
21. Menten B, Pattyn F, De Preter K, et al. arrayCGHbase: an analysis platform for comparative genomic hybridization microarrays. BMC Bioinformatics. 2005;6:124.
22. Coppieters F, Van Schil K, Bauwens M, et al. Identity-by-descent-guided mutation analysis and exome sequencing in consanguineous families reveals unusual clinical and molecular findings in retinal dystrophy. Genet Med. 2014;16(9):671-80.

23. Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA. Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res. 2007;35(Web Server issue):W71-4.
24. Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics. 2008;9:40.
25. Pettersen EF, Goddard TD, Huang CC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem. 2004;25(13):1605-12.
26. Bogaert DJ, De Bruyne M, Debacker V, et al. The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. Haematologica. 2017;102(1):192-202.
27. Dullaers M, Schuijs MJ, Willart M, et al. House dust mite-driven asthma and allergen-specific T cells depend on B cells when the amount of inhaled allergen is limiting. J Allergy Clin Immunol. 2016 Oct 13. pii: S0091-6749(16)31131-9. [Epub ahead of print].
28. Pagano A, Castelnuovo M, Tortelli F, Ferrari R, Dieci G, Cancedda R. New small nuclear RNA gene-like transcriptional units as sources of regulatory transcripts. PLoS Genet. 2007;3(2):e1.
29. Birch J, Clarke CJ, Campbell AD, et al. The initiator methionine tRNA drives cell migration and invasion leading to increased metastatic potential in melanoma. Biol Open. 2016;5(10):1371-9.
30. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol. 1999;93(3):190-7.
31. Shearer WT, Rosenblatt HM, Gelman RS, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol. 2003;112(5):973-80.
32. Piatosa B, Wolska-Kusnierz B, Pac M, Siewiera K, Galkowska E, Bernatowska E. B cell subsets in healthy children: reference values for evaluation of B cell maturation process in peripheral blood. Cytometry B Clin Cytom. 2010;78(6):372-81.
33. Siepel A, Bejerano G, Pedersen JS, et al. Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. Genome Res. 2005;15(8):1034-50.
34. Pollard KS, Hubisz MJ, Rosenbloom KR, Siepel A. Detection of nonneutral substitution rates on mammalian phylogenies. Genome Res. 2010;20(1):110-21.
35. Grantham R. Amino acid difference formula to help explain protein evolution. Science. 1974;185(4154):862-4.
36. Mathe E, Olivier M, Kato S, Ishioka C, Hainaut P, Tavtigian SV. Computational approaches for predicting the biological effect of p53 missense mutations: a comparison of three sequence analysis based methods. Nucleic Acids Res. 2006;34(5):1317-25.
37. Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. Genome Res. 2001;11(5):863-74.
38. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7(4):248-9.
39. Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nat Genet. 2014;46(3):310-5.
40. Itan Y, Shang L, Boisson B, et al. The mutation significance cutoff: gene-level thresholds for variant predictions. Nat Methods. 2016;13(2):109-10.
41. Moens L, Kane A, Tangye SG. Naive and memory B cells exhibit distinct biochemical responses following BCR engagement. Immunol Cell Biol. 2016;94(8):774-86.
42. Bernasconi NL, Traggiai E, Lanzavecchia A. Maintenance of serological memory by polyclonal activation of human memory B cells. Science. 2002;298(5601):2199-202.
43. Recher M, Berglund LJ, Avery DT, et al. IL-21 is the primary common gamma chain-binding cytokine required for human B-cell differentiation in vivo. Blood. 2011;118(26):6824-35.
44. Arpin C, Banchereau J, Liu YJ. Memory B cells are biased towards terminal differentiation: a strategy that may prevent repertoire freezing. J Exp Med. 1997;186(6):931-40.
45. Keller B, Cseresnyes Z, Stumpf I, et al. Disturbed canonical nuclear factor of kappa light chain signaling in B cells of patients with common variable immunodeficiency. J Allergy Clin Immunol. 2017;139(1):220-231.e8.

46. van de Ven AA, Hoytema van Konijnenburg DP, Wensing AM, van Montfrans JM. The role of prolonged viral gastrointestinal infections in the development of immunodeficiency-related enteropathy. Clin Rev Allergy Immunol. 2012;42(1):79-91.
47. Kamphuis E, Junt T, Waibler Z, Forster R, Kalinke U. Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia. Blood. 2006;108(10):3253-61.
48. Tangye SG, Avery DT, Deenick EK, Hodgkin PD. Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. J Immunol. 2003;170(2):686-94.
49. Trickett A, Kwan YL. T cell stimulation and expansion using anti-CD3/CD28 beads. J Immunol Methods. 2003;275(1-2):251-5.
50. Ceuppens JL, Baroja ML, Lorre K, Van Damme J, Billiau A. Human T cell activation with phytohemagglutinin. The function of IL-6 as an accessory signal. J Immunol. 1988;141(11):3868-74.
51. Moore JBt, Farrar JE, Arceci RJ, Liu JM, Ellis SR. Distinct ribosome maturation defects in yeast models of Diamond-Blackfan anemia and Shwachman-Diamond syndrome. Haematologica. 2010;95(1):57-64.
52. Golomb L, Volarevic S, Oren M. p53 and ribosome biogenesis stress: the essentials. FEBS Lett. 2014;588(16):2571-9.
53. Orelio C, Verkuijlen P, Geissler J, van den Berg TK, Kuijpers TW. SBDS expression and localization at the mitotic spindle in human myeloid progenitors. PLoS One. 2009;4(9):e7084.
54. Austin KM, Gupta ML, Jr., Coats SA, et al. Mitotic spindle destabilization and genomic instability in Shwachman-Diamond syndrome. J Clin Invest. 2008;118(4):1511-8.
55. Ammons D, Rampersad J. An E. coli 5S rRNA deletion mutant useful for the study of 5S rRNA structure/function relationships. Curr Microbiol. 2001;43(2):89-92.
56. Onofrillo C. Ribosome biogenesis and cell cycle regulation: effect of RNA polymerase III inhibition. PhD dissertation. Italy, Universita di Bologna; 2013.
57. Danilova N, Gazda HT. Ribosomopathies: how a common root can cause a tree of pathologies. Dis Model Mech. 2015;8(9):1013-26.
58. Cong L, Ran FA, Cox D, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339(6121):819-23.
59. Schumann K, Lin S, Boyer E, et al. Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. Proc Natl Acad Sci U S A. 2015;112(33):10437-42.
60. Louis AG, Gupta S. Primary selective IgM deficiency: an ignored immunodeficiency. Clin Rev Allergy Immunol. 2014;46(2):104-11.
61. Durandy A, Kracker S, Fischer A. Primary antibody deficiencies. Nat Rev Immunol. 2013;13(7):519-33.
62. Bousfiha A, Jeddane L, Al-Herz W, et al. The 2015 IUIS Phenotypic Classification for Primary Immunodeficiencies. J Clin Immunol. 2015;35(8):727-38.
63. Stohl W, Elliot JE. In vitro inhibition by intravenous immunoglobulin of human T cell-dependent B cell differentiation induced by staphylococcal superantigens. Clin Immunol Immunopathol. 1996;79(2):122-33.

Acknowledgements

The authors gratefully acknowledge the patients and their family who participated in this study. We thank Veronique Debacker for her assistance in patient sample collection.

This study was supported by the Ghent University Hospital Spearhead Initiative for Immunology Research, the Jeffrey Modell Foundation to F.H., the Research Foundation Flanders (FWO) to D.B., T.K. and E.D.B., the Ghent University Special Research Fund (BOF15/GOA/011) to E.D.B., Hercules foundation AUGE/13/023 to E.D.B., and the NIH Clinical Center intramural research program to H.S.K. and S.D.R. D.B. is a PhD fellow and T.K. and E.D.B. are Senior Clinical Investigators of the FWO.

Chapter 8

General discussion and future perspectives

8 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Common variable immunodeficiency (CVID) is one of the most frequently diagnosed primary immunodeficiencies (PIDs) with an estimated prevalence between 1/100,000 and 1/10,000.[1,2] This means that in Belgium, with a population of about 11.5 million, between 115 and 1150 individuals suffer from CVID. Although CVID is a rare disease, it carries a high burden to patients and their families as well as to healthcare providers and society. Recurrent and/or severe infections are a major cause of morbidity in CVID patients, and disease-related complications can lead to an 11-fold increase of mortality.[3,4] Furthermore, healthcare providers are faced with a high number of medical encounters while universal guidelines on follow-up and treatment of disease-related complications are lacking.[5] Finally, frequent healthcare visits, repeated hospitalizations and, especially, life-long treatment with expensive immunoglobulin preparations put pressure on society's healthcare budgets.[6]

It has become apparent that CVID is an umbrella diagnosis covering a clinically, immunologically and genetically heterogeneous group of disorders.[7,8] To date, the pathogenesis of CVID remains largely enigmatic and a monogenic defect has only been identified in 2-10% of patients.[7,8] In addition, patients with primary antibody deficiencies (PADs) resembling CVID but not reaching the somewhat arbitrary diagnostic cutoffs of reduced serum immunoglobulin (Ig) levels, have been less well studied.[9,10]

In this thesis, we aimed to utilize detailed immunophenotypical characterization to facilitate the identification of (novel) molecular genetic defects in a cohort of patients diagnosed with CVID or related PADs mainly including idiopathic primary hypogammaglobulinemia (IPH) and IgG subclass deficiency (IgGSD).

Summarizing discussion on the main findings of this thesis

In **chapter 3**, detailed immunological characterization of peripheral blood mononuclear cells (PBMCs) in patients with CVID, IPH and IgGSD demonstrated that some immunophenotypical alterations were shared between all PADs whereas others were only associated with CVID. Unexpectedly, asymptomatic first-degree family members of patients (AFM) had lower mean levels of serum IgG and IgM than healthy controls and showed similar, albeit less pronounced, abnormalities in peripheral B and T cell subsets than PAD patients. Hence, by simultaneously investigating a broad range of parameters in three groups of PAD patients and their AFM, we were able to show for the first time in a larger cohort that peripheral immunophenotypical abnormalities of PADs form a spectrum ranging from AFM over IgGSD and IPH patients to CVID patients, being most notable in naive and memory B and T cell subsets. Moreover, unsupervised computational clustering techniques were

unable to discriminate subgroups of subjects, further underlining the heterogeneous and overlapping nature of CVID and related PADs. Together, our findings support recent notions that the majority of CVID(-like) patients have a complex rather than a monogenic etiology, whereby an accumulation of multiple genetic, epigenetic and/or environmental factors contributes to the final phenotype.[8,11,12]

As outlined in **chapter 4**, we so far identified a causative monogenic defect in seven out of 70 index patients (10%) from our PAD cohort. In five of those patients, hallmark immunological abnormalities (*BTK*, *GATA2*) or the presence of extra-immunological features (18q deletion, *RNU4ATAC*, *KMT2A*) provided additional clues towards the identification of the underlying genetic defect. These "clues" were sometimes not yet apparent at time of inclusion in the study, however. The mutations in the other two patients were detected by an exploratory approach, either via targeted resequencing (*NFKB1*) or whole exome sequencing (WES) (*IKZF1*). In our cohort, WES had a considerably higher detection rate (15%) than targeted sequencing of known disease genes (5.7%). Moreover, WES also revealed three novel candidate disease genes, for which functional validation is currently ongoing or planned in the near future. We therefore concur with the literature that WES and WGS, which was not performed in this study, are currently the preferred approach for genetic testing in patients with CVID and related PADs.[13,14] When drawing up the balance sheet of genetic testing in our PAD cohort, three compelling observations were made. First, in two of the 7 solved index cases, the causal mutation was located in **non-coding regions**: in an intronic region of a protein-coding gene (*GATA2*) and in a non-coding RNA gene (*RNU4ATAC*), respectively. Although *GATA2* and *RNU4ATAC* are not regarded as CVID-associated genes, this observation suggests that non-coding variation may be able to explain a subgroup of patients diagnosed with CVID or related PAD disorders. The role of non-coding variation in these conditions is, however, an underexplored but emerging field given the implementation of WGS in the near future. Second, phenotype-based targeted sequencing of candidate genes had a yield of about 5.7% in our cohort, which is markedly lower than what is expected in Mendelian diseases.[15] This reflects the **lack of a phenotype-genotype correlation** for many CVID- and in extension PID-associated genes.[16] Third, disease-causing mutations were also found in 4% of asymptomatic family members of index patients, not including unaffected carriers of recessive alleles. These last two observations support the idea that **CVID and related PADs are mostly non-Mendelian phenotypes** influenced by the actions of modifier genes, epigenetic changes and/or environmental factors.[8,17]

In **the following chapters of this thesis**, we focused on three families with novel or rare variants in known disease genes that expanded phenotypical spectra and one family with a novel candidate disease gene. The two sibling pairs with *KMT2A*-associated Wiedemann-Steiner syndrome (WSS)[18] and *RNU4ATAC*-associated Roifman syndrome (RS)[19],

respectively, described in **chapter 5**, stress the importance of a thorough genetic assessment in patients presenting with a CVID-reminiscent phenotype at a very young age, including proactive evaluation for extra-immunological syndromic features. In addition, we found reduced levels of circulating follicular helper T (cTfh) cells in all WSS and RS patients, and decreased BAFF-R expression on B cells in the RS patients. These immunological abnormalities had not been previously reported and were in line with the impaired B cell differentiation and/or decreased total B cell numbers, respectively, seen in the patients. It will be of interest to investigate these parameters in additional patients with WSS and RS as they may help elucidate the underlying pathophysiology of primary antibody failure in these syndromic disorders.

In **chapter 6**, we examined the first truncating *IKZF1* mutation associated with IKAROS haploinsufficiency in two symptomatic sisters and their asymptomatic mother.[20-22] The premature stop codon near the N-terminal end was particularly interesting because *IKZF1* is believed to be under strong purifying selection, meaning that deleterious alleles are usually purged from the population.[22] In accordance, only one premature stop codon in *IKZF1* (variant 7: 50468298 AG/A), located near the C-terminal end, was found in more than 126,000 exomes and 15,000 genomes included in gnomAD Browser, currently the largest public database (http://gnomad.broadinstitute.org). However, the variable expressivity and incomplete penetrance seen in previously published patients with germline IKAROS haploinsufficiency indicate that additional disease-influencing mechanisms are at play.[22] The three *IKZF1* mutation carriers of the here-examined kindred showed similarly low IKAROS protein expression levels but a remarkable variation in clinical involvement and B cell maturation defects. They demonstrated a variable, multi-level central B cell developmental block situated at a later stage than the block observed in four previously reported symptomatic subjects. Since these previous cases all harbored missense mutations in the same protein domain, a possible genotype-phenotype correlation should be further explored.[21,22] Interestingly, the reduced central B-lineage cells in the asymptomatic mother were, so far, able to sustain normal peripheral B cell development and numbers.[21,22]

Finally, in **chapter 7**, we investigated novel compound heterozygous variants in *GTF3A*, encoding transcription factor IIIA (TFIIIA),[23] identified in a Belgian brother and sister pair with a CVID-like phenotype. *GTF3A* has not been previously implicated in disease and a possible role of TFIIIA in the immune system is unclear. Notwithstanding, *in silico* pathogenicity prediction tools and protein modeling as well as preliminary functional validation studies have provided evidence that the *GTF3A* variants could be disease-causing through loss of TFIIIA function. Since TFIIIA regulates *5S rRNA* transcription important for ribosome biogenesis[24-26], we hypothesize that biallelic loss-of-function *GTF3A* mutations cause a novel ribosomopathy

featured by CVID-like antibody failure.[27,28] Future studies are planned to investigate a direct causal relationship between the *GTF3A* genotype and the phenotype in these patients.

The utility of extensive immunophenotyping to guide genetic testing and WES data mining

The identification of genetic defects in patients diagnosed with CVID has proven to be arduous.[13] Thus far, monogenic defects have been predominantly identified in patients with an early disease onset, disease-related complications such as autoimmunity or lymphoproliferation, a positive family history, and/or consanguineous parents.[8,13] As mentioned above, the main objective of this thesis was to utilize extensive peripheral blood immunophenotyping to facilitate the discovery of genetic defects in our PAD cohort. This objective was built on the fact that the peripheral blood compartment is far more easily accessible than lymphoid organs, increasing the likelihood of participation in the study, and that white blood cell constituents mirror (ab)normal immunological processes in the body. Detailed immunophenotyping of PBMCs could, therefore, indicate which immunological pathways might be affected. However, we found that the latter was only partly true. In general, a first "basic" immunophenotypical evaluation of the patients was performed in a routine clinical setting, including total B, total/$CD4^+$/$CD8^+$ T, and total NK lymphocyte counts, naive and memory T cell subsets, and naive, transitional, marginal zone-like, switched memory and $CD21^{low}$ B cell subsets. Upon inclusion in the study, more extensive immunophenotyping (as explained in **chapter 3**) was performed in research setting. Based on our experience from this project, we conclude that more extensive immunophenotyping did not have an added value to routine lymphocyte phenotyping for the first phases of genetic testing. In other words, "basic" immunophenotypical information was necessary and sufficient for an initial genetic assessment. An important footnote is that numerous study participants had not undergone a routine immunophenotypical evaluation since this is not required for a diagnosis of CVID or related PADs and is of limited prognostic value.[7,29] From this experience we learn that future genetic studies in PAD patients would not only benefit from a complete clinical workup, but also from complete lymphocyte phenotyping in routine setting. In rare cases, (immuno)phenotypical information can directly point to one or a few specific genotypes. For example, in our patient cohort, the combination of clinical features with routine immunophenotyping eventually led to the identification of mutations in *BTK*, *GATA2*, and *RNU4ATAC* through targeted sequencing. Furthermore, to analyze WES data, the investigator must know what he is looking for, at least to certain extent, to be able to filter on biologically relevant variants. More specialized immunological investigations can afterwards be performed in function of genetic variants of interest. Overall, genetic studies in

heterogeneous disorders like CVID and related PADs ask for an interactive collaboration between clinicians, routine laboratory immunologists and geneticists, and researchers.[14]

The importance of a genetic diagnosis

Like for all monogenic disorders, identifying a monogenic subtype of CVID supports the clinical diagnosis, genetic counseling of the index case and his/her family and reproductive options. Establishing a genetic diagnosis can also entail important implications in terms of patient management. When the gene has already been described in other patients, knowing the genetic defect helps to assess the prognosis of the patient and to guide follow-up with screening for specific complications.[8,14]

Immunoglobulin replacement therapy, the current cornerstone of treatment, significantly decreases the infection frequency but has little effect on disease-related non-infectious complications.[7] Therefore, other treatment modalities are being explored. In some monogenic subtypes (e.g. *LRBA*, *PIK3CD*), hematopoietic stem cell transplantation (HSCT) is now considered an acceptable treatment option.[30,31] In clinical practice, the decision to perform high-risk HSCT in PID patients with a severe clinical course often hinges on the identification of the underlying genetic defect, underlining the importance of an early genetic diagnosis. Furthermore, the identification of monogenic defects has offered the opportunity to develop targeted drug therapies.[32] As mentioned in the introduction of this thesis (section 1.7), a few targeted immunomodulatory drugs have already been reported to be of potential use: the CTLA-4 mimetic abatacept in LRBA and CTLA-4 deficiencies, and mTOR inhibitors in patients with activated phosphatidyl-inositol-3-kinase (PI3K) p110δ syndrome (APDS).[33-35] Two clinical trials of PI3K p110δ-specific inhibitors in patients with APDS have been recently announced.[36] Note that targeted drug therapies are also being explored in many other PID disorders.[32] Another major opportunity of a molecular diagnosis is gene-based therapy. Gene therapy relying on viral vectors to transfer a functional copy of the mutated gene to autologous HSCs has shown great promise in selected PID disorders.[38] The newer SIN-gammaretroviral and lentiviral vector platforms are more effective and markedly safer than the first generation of gammaretroviral vectors. In recent years, several gene-editing technologies have been developed such as zinc finger nucleases (ZNF), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats/CRISPR-associated gene 9 (CRISPR/Cas9).[38] In contrast to vector-based gene addition platforms, gene-editing approaches can precisely target and repair a mutated genetic sequence, or can precisely target an insertion region within the genome enabling the use of natural regulatory elements and decreasing the risk of insertional mutagenesis. The application of gene-editing strategies in PIDs is currently being

investigated.[38] Although gene-based therapies for PIDs are still mostly experimental, in 2016 the European Commission approved a viral vector gene therapy for adenosine deaminase deficient severe combined immunodeficiency (ADA-SCID) called Strimvelis.[39] It is both the first gammaretrovirus-based and the first *ex vivo* gene therapy to be granted marketing authorization. This approval is based on a clinical trial in 18 children that has shown a 100% survival rate with substantial immunological improvement and no leukemic events up to 13 years after treatment.[39] In the future, gene-based therapy may become a valid treatment option for all monogenic PIDs in which case a genetic diagnosis will become indispensible.
Finally, the identification of monogenic defects in CVID, and PIDs in general, has important implications for the field of basic immunology as it expands our understanding on basic cellular pathways and mechanisms of host defense.[40] Unraveling fundamental immunological mechanisms in humans has also revealed striking differences with animal models, indicating the importance of human studies and PIDs as "experiments of nature".[40] Novel insights in basic immunology can, in turn, be translated back into clinical immunology to continue optimization of patient care.[40]

Future directions in investigating the molecular basis of CVID

Studies on the molecular basis of CVID have mainly focused on identifying monogenic defects. Still, a few genome-wide and epigenetic studies have addressed the hypothesis of a complex polygenic or multifactorial molecular basis of CVID. As discussed in the introduction of this thesis (section 1.6.3.3), two GWAS of CVID cohorts have reported several significantly associated loci.[41,42] In the first GWAS, the authors used the SNP dataset to build an algorithmic model for predicting the risk of developing CVID.[41] Although the internal validation test was successful and identified CVID patients with 98,7% accuracy, the prediction model has, to our knowledge, not been used in other study cohorts.[41] In addition, the first GWAS reported a significantly higher burden of CNVs in CVID patients compared to controls.[41] So far, CNVs have not been systematically examined in CVID patients. A recent report on 278 PID index patients has detected several PID-causing CNVs of which some were intragenic CNVs smaller than 30 kb that would have been missed with the genome-wide array CGH used here (resolution ~100 kb).[14] The second GWAS found that CVID shares genetic susceptibility loci with complex autoimmune diseases such as the locus containing the *CLEC16A* gene.[42] A common genetic basis could be one of the keys to further unravel the co-occurrence of immunodeficiency and autoimmunity seen in many CVID patients.[17] van Schouwenburg *et al.* performed WGS and RNA sequencing in sporadic CVID cases.[11] Despite the relatively small sample size (n=32), the lack of functional validation analyses and absence of replication in other cohorts, this first WGS-based study in a CVID

cohort has generated interesting hypotheses that should be further explored.[11] The authors found that all patients showed evidence of a mutational burden in shared genes and pathways implicated in B cell biology.[11] Future pathway and network analyses in larger CVID cohorts would be of interest to determine the collective contribution of genetic variants that individually do not suffice to cause disease.[17] Furthermore, the combination of WGS and RNA sequencing in three sporadic CVID cases has provided proof-of-concept that non-coding variation and differential gene expression could play a role in the pathogenesis of CVID.[11] DNA methylation is one of the mechanisms of epigenetic regulation of gene expression.[43] Differences in DNA methylation and subsequent gene expression have recently been identified in B cells derived from monozygotic twins discordant for CVID.[12] Thus, epigenetic mechanisms may also be involved in the etiology of CVID.[12] Epigenetic processes are, in turn, influenced by environmental factors such as smoking, diet and perhaps even infectious agents.[43] Indeed, similar to other complex diseases, the risk of developing CVID may be modified by an interplay between internal genetic components and environmental influences.[17]

In summary, genomics (including analysis of structural variations such as CNVs, non-coding variation, and pathway and network analyses of possible disease-modifying variants), epigenomics, transcriptomics, and proteomics will form the garrison for future studies on the molecular basis of CVID. Although much of the generated data will be challenging to interpret, it could offer important insights in the underlying disease-mechanisms of CVID at different molecular levels.[17] Moreover, the integration of multi-level molecular data could significantly enhance our understanding on the etiology of CVID.[17] In addition, multi-level molecular data could offer new opportunities for distinguishing subgroups of CVID patients and establishing reliable biomarkers to predict the risk of developing disease-related complications.[17]

Has the term "CVID" become obsolete?

The diagnosis of CVID is an umbrella for a wide range of distinct disorders. Hence, one might ask if the term "CVID" has become obsolete in the era of advanced genomic and molecular technologies. In my view, the answer to this question is "no, not at all". A first argument is, of course, the fact that currently identified monogenic defects only encompass 2-10% of patients diagnosed with CVID. Secondly, since genetic testing is not yet readily available in the consultation room, but, in contrast, can still take a lengthy period of time, it is essential to have a clinical diagnosis. A clinical diagnosis is not only the starting point for patient management decisions, but is also important for administrative purposes such as reimbursement requests for immunoglobulin replacement therapy, claims for higher child

benefits, and patient registration. Lastly, if CVID indeed has a multifactorial basis in the majority of patients, this term will probably remain to exist to cover that group of patients while those with a monogenic defect will be removed from under the umbrella.

Instead of questioning the continued existence of the term “CVID”, I believe that a change in mentality on how and when to use this term is more at hand. In particular, we should increase awareness among healthcare providers and scientists that a diagnosis of CVID is not the end point of the diagnostic process, and that continuous re-evaluation and, if possible, molecular (genetic) testing should be pursued. For now, a definitive molecular diagnosis will not be reached in many CVID patients, but with the current evolution in technological advances, the future of precision medicine in CVID looks more promising than ever.[40]

References

1. Chapel H, Cunningham-Rundles C. Update in understanding common variable immunodeficiency disorders (CVIDs) and the management of patients with these conditions. Br J Haematol. 2009;145(6):709-27.
2. Modell V, Quinn J, Orange J, Notarangelo LD, Modell F. Primary immunodeficiencies worldwide: an updated overview from the Jeffrey Modell Centers Global Network. Immunol Res. 2016;64(3):736-53.
3. Resnick ES, Moshier EL, Godbold JH, Cunningham-Rundles C. Morbidity and mortality in common variable immune deficiency over 4 decades. Blood. 2012;119:1650-7.
4. Quinti I, Soresina A, Spadaro G, et al. Long-term follow-up and outcome of a large cohort of patients with common variable immunodeficiency. J Clin Immunol. 2007;27(3):308-16.
5. Abolhassani H, Sagvand BT, Shokuhfar T, Mirminachi B, Rezaei N, Aghamohammadi A. A review on guidelines for management and treatment of common variable immunodeficiency. Expert Rev Clin Immunol. 2013;9(6):561-74; quiz 575.
6. Sadeghi B, Abolhassani H, Naseri A, Rezaei N, Aghamohammadi A. Economic burden of common variable immunodeficiency: annual cost of disease. Expert Rev Clin Immunol. 2015;11(5):681-8.
7. Bonilla FA, Barlan I, Chapel H, et al. International Consensus Document (ICON): Common Variable Immunodeficiency Disorders. J Allergy Clin Immunol Pract. 2016;4(1):38-59.
8. Bogaert DJ, Dullaers M, Lambrecht BN, Vermaelen KY, De Baere E, Haerynck F. Genes associated with common variable immunodeficiency: one diagnosis to rule them all? J Med Genet. 2016;53(9):575-90.
9. Ameratunga R, Woon ST, Gillis D, Koopmans W, Steele R. New diagnostic criteria for common variable immune deficiency (CVID), which may assist with decisions to treat with intravenous or subcutaneous immunoglobulin. Clin Exp Immunol. 2013;174(2):203-11.
10. Bertinchamp R, Gerard L, Boutboul D, Malphettes M, Fieschi C, Oksenhendler E. Exclusion of Patients with a Severe T-Cell Defect Improves the Definition of Common Variable Immunodeficiency. J Allergy Clin Immunol Pract. 2016;4(6):1147-57.
11. van Schouwenburg PA, Davenport EE, Kienzler AK, et al. Application of whole genome and RNA sequencing to investigate the genomic landscape of common variable immunodeficiency disorders. Clin Immunol. 2015;160(2):301-14.
12. Rodriguez-Cortez VC, Del Pino-Molina L, Rodriguez-Ubreva J, et al. Monozygotic twins discordant for common variable immunodeficiency reveal impaired DNA demethylation during naive-to-memory B-cell transition. Nat Commun. 2015;6:7335.
13. Maffucci P, Filion CA, Boisson B, et al. Genetic Diagnosis Using Whole Exome Sequencing in Common Variable Immunodeficiency. Front Immunol. 2016;7:220.
14. Stray-Pedersen A, Sorte HS, Samarakoon P, et al. Primary immunodeficiency diseases: Genomic approaches delineate heterogeneous Mendelian disorders. J Allergy Clin Immunol. 2017;139(1):232-45.
15. Liu Y, Wei X, Kong X, et al. Targeted Next-Generation Sequencing for Clinical Diagnosis of 561 Mendelian Diseases. PLoS One. 2015;10(8):e0133636.
16. Meyts I, Bosch B, Bolze A, et al. Exome and genome sequencing for inborn errors of immunity. J Allergy Clin Immunol. 2016;138(4):957-69.
17. Li J, Wei Z, Li YR, et al. Understanding the genetic and epigenetic basis of common variable immunodeficiency disorder through omics approaches. Biochim Biophys Acta. 2016;1860(11 Pt B):2656-63.
18. Stellacci E, Onesimo R, Bruselles A, et al. Congenital immunodeficiency in an individual with Wiedemann-Steiner syndrome due to a novel missense mutation in KMT2A. Am J Med Genet A. 2016;170(9):2389-93.
19. Merico D, Roifman M, Braunschweig U, et al. Compound heterozygous mutations in the noncoding RNU4ATAC cause Roifman Syndrome by disrupting minor intron splicing. Nat Commun. 2015;6:8718..

20. Goldman FD, Gurel Z, Al-Zubeidi D, et al. Congenital pancytopenia and absence of B lymphocytes in a neonate with a mutation in the Ikaros gene. Pediatr Blood Cancer. 2012;58(4):591-7.
21. Hoshino A, Okada S, Yoshida K, et al. Abnormal hematopoiesis and autoimmunity in human subjects with germline IKZF1 mutations. J Allergy Clin Immunol. 2016. pii: S0091-6749(16)31273-8. [Epub ahead of print]
22. Kuehn HS, Boisson B, Cunningham-Rundles C, et al. Loss of B Cells in Patients with Heterozygous Mutations in IKAROS. N Engl J Med. 2016;374(11):1032-43.
23. Moorefield B, Roeder RG. Purification and characterization of human transcription factor IIIA. J Biol Chem. 1994;269(33):20857-65.
24. Engelke DR, Ng SY, Shastry BS, Roeder RG. Specific interaction of a purified transcription factor with an internal control region of 5S RNA genes. Cell. 1980;19(3):717-28.
25. Oler AJ, Alla RK, Roberts DN, et al. Human RNA polymerase III transcriptomes and relationships to Pol II promoter chromatin and enhancer-binding factors. Nat Struct Mol Biol. 2010;17(5):620-8.
26. Donati G, Peddigari S, Mercer CA, Thomas G. 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. Cell Rep. 2013;4(1):87-98.
27. Khan S, Pereira J, Darbyshire PJ, et al. Do ribosomopathies explain some cases of common variable immunodeficiency? Clin Exp Immunol. 2011;163(1):96-103.
28. Yelick PC, Trainor PA. Ribosomopathies: Global process, tissue specific defects. Rare Dis. 2015;3(1):e1025185.
29. Chapel H. Common Variable Immunodeficiency Disorders (CVID) - Diagnoses of Exclusion, Especially Combined Immune Defects. J Allergy Clin Immunol Pract. 2016;4(6):1158-9.
30. Coulter TI, Chandra A, Bacon CM, et al. Clinical spectrum and features of activated phosphoinositide 3-kinase delta syndrome: A large patient cohort study. J Allergy Clin Immunol. 2017;139(2):597-606.e4.
31. Seidel MG, Hirschmugl T, Gamez-Diaz L, et al. Long-term remission after allogeneic hematopoietic stem cell transplantation in LPS-responsive beige-like anchor (LRBA) deficiency. J Allergy Clin Immunol. 2015;135(5):1384-90.e1-8.
32. Vignesh P, Rawat A, Singh S. An Update on the Use of Immunomodulators in Primary Immunodeficiencies. Clin Rev Allergy Immunol. 2017;52(2):287-303.
33. Lucas CL, Kuehn HS, Zhao F, et al. Dominant-activating germline mutations in the gene encoding the PI(3)K catalytic subunit p110delta result in T cell senescence and human immunodeficiency. Nat Immunol. 2014;15(1):88-97.
34. Lo B, Zhang K, Lu W, et al. AUTOIMMUNE DISEASE. Patients with LRBA deficiency show CTLA4 loss and immune dysregulation responsive to abatacept therapy. Science. 2015;349(6246):436-40.
35. Lee S, Moon JS, Lee CR, et al. Abatacept alleviates severe autoimmune symptoms in a patient carrying a de novo variant in CTLA-4. J Allergy Clin Immunol. 2016;137(1):327-30.
36. Lucas CL, Chandra A, Nejentsev S, Condliffe AM, Okkenhaug K. PI3Kdelta and primary immunodeficiencies. Nat Rev Immunol. 2016;16(11):702-14.
37. Rizzi M, Neumann C, Fielding AK, et al. Outcome of allogeneic stem cell transplantation in adults with common variable immunodeficiency. J Allergy Clin Immunol. 2011;128(6):1371-1374.e2.
38. Thrasher AJ, Williams DA. Evolving gene therapy in primary immunodeficiency. Mol Ther. 2017;25(5):1132-41.
39. Schimmer J, Breazzano S. Investor Outlook: Rising from the ashes; GSK's European approval of Strimvelis for ADA-SCID. Hum Gene Ther Clin Dev. 2016;27(2):57-61.
40. Milner JD, Holland SM. The cup runneth over: lessons from the ever-expanding pool of primary immunodeficiency diseases. Nat Rev Immunol. 2013;13(9):635-48.
41. Orange JS, Glessner JT, Resnick E, et al. Genome-wide association identifies diverse causes of common variable immunodeficiency. J Allergy Clin Immunol. 2011;127(6):1360-7.e6.
42. Li J, Jorgensen SF, Maggadottir SM, et al. Association of CLEC16A with human common variable immunodeficiency disorder and role in murine B cells. Nat Commun. 2015;6:6804.

43. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003;33 Suppl:245-54.

Summary

Samenvatting

SUMMARY

Common variable immunodeficiency (CVID) is one of the most prevalent primary immunodeficiencies (PIDs) estimated to affect 1/100,000 to 1/10,000 people worldwide. CVID is a collective term for a clinically, immunologically and genetically heterogeneous group of disorders characterized by markedly reduced serum immunoglobulin levels, inadequate antibody responses to vaccinations, recurrent infections, and complications due to immune dysregulation (e.g. autoimmunity, malignancy). The molecular pathogenesis of CVID remains largely unknown. Up to date, a monogenic cause is only found in 2-10% of patients. Accumulating data suggest that most CVID patients have a multifactorial etiology, meaning that multiple genetic, epigenetic and/or environmental factors determine the final phenotype. In extension, primary antibody deficiencies (PADs) showing phenotypical overlap with CVID but with a less severely impaired antibody production may be part of the same multifactorial disease spectrum. The general aim of this thesis was to unravel the underlying monogenic defects in patients with CVID and related PADs followed in our center by using an integrative immunological and genetic approach.

We started by investigating the peripheral blood immunological phenotype of 77 patients with CVID and two milder PAD disorders named idiopathic primary hypogammaglobulinemia (IPH) and IgG subclass deficiency (IgGSD), as well as 47 asymptomatic first-degree family members of patients (AFM) and 101 age-matched healthy controls (HC) (**chapter 3**). Interestingly, some immunophenotypical alterations were shared between all three patient groups whereas others were only associated with CVID. Unexpectedly, AFM showed similar, albeit intermediate, abnormalities in peripheral B and T cell subsets compared to PAD patients. Moreover, unsupervised computational clustering techniques were unable to discriminate subgroups of subjects in our cohort. Together, we demonstrated for the first time in a relatively large cohort that peripheral immunophenotypical abnormalities in PADs form a spectrum ranging from AFM over milder disorders like IgGSD and IPH to the most severe disorder CVID. Our findings add to the growing body of literature on the complex etiological basis of CVID and related PADs.

Chapter 4 describes the genetic analyses performed in our PAD cohort using microarray-based comparative genomic hybridization, Sanger sequencing, targeted next-generation sequencing (NGS), whole exome sequencing (WES), and homozygosity mapping. So far, a disease-causing genetic defect was identified in 7 out of 70 index patients (10%) from our cohort: three patients with a syndromic form of PAD (caused by a 18q deletion, *KMT2A* or *RNU4ATAC* mutations respectively), one patient with agammaglobulinemia (caused by a

BTK mutation), and three patients with a prior diagnosis of IPH or CVID (caused by *GATA2*, *NFKB1*, or *IKZF1* mutations respectively). In five of the seven solved cases, hallmark (extra-) immunological features ultimately provided essential clues. Remarkably, targeted sequencing of known disease genes had a yield of only 5.7% (4/70). WES revealed novel mutations in known disease genes in two out of 13 tested index patients (15%), known disease-modifying variants in four patients (31%), and novel candidate disease genes in three cases (23%). Genetic analyses in this cohort are still ongoing.

In **chapter 5**, we studied a family with a novel heterozygous mutation in *KMT2A* causing Wiedemann-Steiner syndrome (WSS), and a second family with rare compound heterozygous mutations in the non-coding RNA gene *RNU4ATAC* causing Roifman syndrome (RS). In both families, the sibling pairs exceptionally presented with a CVID phenotype in early childhood while extra-immunological features were not apparent at that time, significantly delaying the establishment of an accurate diagnosis. This underlines the importance of pursuing a genetic diagnosis in patients with an early-onset CVID phenotype. In addition, we found previously unreported abnormalities in circulating follicular helper T (cTfh) cells in WSS and RS, and in B cell BAFF-R expression in RS, suggestive for novel pathophysiogical insights in these syndromic disorders.

We next examined two patients and their mother who harbored a novel heterozygous mutation in *IKZF1* leading to IKAROS haploinsufficiency (**chapter 6**). All three subjects had a variable, multi-level central B cell developmental block which was situated more downstream compared to the arrest observed in previously published symptomatic cases. Although the mother had reduced bone marrow B-lineage cells, peripheral blood B cell subsets were within normal ranges and clinical symptoms were absent. With this study, we provided novel insights in central B cell development in both symptomatic and asymptomatic *IKZF1* mutation carriers.

Finally, in **chapter 7** we identified a novel candidate disease gene, *GTF3A*. In particular, we found novel compound heterozygous variants in *GTF3A* in a Belgian brother and sister pair with a CVID-like phenotype and profound B cell proliferation defects. Preliminary functional validation studies have provided evidence for pathogenicity of the *GTF3A* variants through loss-of-function. Since *GTF3A* has a known role in ribosome biogenesis and some ribosomopathies have been previously associated with various lymphocyte abnormalities, we hypothesize that biallelic *GTF3A* mutations cause a novel ribosomopathy characterized by a CVID-like phenotype. Future studies are planned to explore a direct causal relationship between the *GTF3A* genotype and the phenotype in the patients.

SAMENVATTING

Common variable immunodeficiency (CVID) is één van de meest voorkomende primaire immuundeficiënties (PIDs) en treft naar schatting 1/100.000 tot 1/10.000 mensen wereldwijd. CVID is een verzamelterm voor een klinisch, immunologisch en genetisch heterogene groep van aandoeningen gekenmerkt door sterk verlaagde immuunglobuline serumspiegels, onvoldoende vorming van antistoffen na vaccinaties, herhaaldelijke infecties, en complicaties ten gevolge van ontregeling van het immuunsysteem (bijv. autoimmuniteit, maligniteit). De moleculaire pathogenese van CVID is nog grotendeels onduidelijk. Op dit moment wordt een monogenetische oorzaak slechts in 2-10% van de patiënten gevonden. Recente studies suggereren dat de meeste CVID patiënten een multifactoriële etiologie vertonen, waarbij meerdere genetische, epigenetische en/of omgevingsfactoren het uiteindelijke fenotype bepalen. Aansluitend kunnen we stellen dat primaire antilichaamdeficiënties (PADs) die fenotypisch gelijken op CVID maar met een minder uitgesproken antilichaamstoornis, mogelijk deel uitmaken van hetzelfde multifactoriële ziektespectrum. Het algemene doel van deze thesis was om bij patiënten met CVID en gerelateerde PADs, die gevolgd worden in ons centrum, het onderliggende monogenetische defect te identificeren aan de hand van een geïntegreerde immunologische en genetische aanpak.

In een eerste fase hebben we op perifeer bloed het immunologisch fenotype nagekeken bij 77 patiënten met CVID en twee mildere PAD aandoeningen, namelijk idiopathische primaire hypogammaglobulinemie (IPH) en IgG subklasse deficiëntie (IgGSD), alsook bij 47 asymptomatische eerstegraadsverwanten van patiënten (AFM) en 101 leeftijdsgematchte gezonde controles (HC) (**hoofdstuk 3**). Bepaalde immunofenotypische veranderingen werden gedetecteerd in de drie patiëntengroepen terwijl andere afwijkingen enkel geassocieerd waren met CVID. Verrassend stelden we vast dat AFM gelijkaardige, al zij het intermediaire, afwijkingen vertoonden in perifere B en T cel subsets in vergelijking met PAD patiënten. Daarnaast konden ongecontroleerde computationele clustertechnieken geen subgroepen van studiepersonen onderscheiden in onze cohorte. Samenvattend hebben we voor het eerst in een relatief grote cohorte aangetoond dat perifere immunofenotypische afwijkingen in PADs een spectrum vormen gaande van AFM over mildere aandoeningen zoals IgGSD en IPH tot de ernstigste aandoening CVID. Deze bevindingen dragen bij tot recente literatuur over de complexe etiologie van CVID en gerelateerde PADs.

Hoofdstuk 4 beschrijft de genetische analyses die uitgevoerd werden op onze PAD cohorte, waarbij gebruik gemaakt werd van microrooster-gebaseerde vergelijkende

genoomhybridisatie, Sanger sequenering, doelgerichte *next-generation* sequenering (NGS), volledige exoom sequenering (WES), en homozygositeitsmapping. Tot nu toe werd een ziekte-veroorzakend genetisch defect geïdentificeerd in 7 van de 70 index patiënten (10%) van onze cohorte: drie patiënten met een syndromale vorm van PAD (respectievelijk door een 18q deletie, *KMT2A* of *RNU4ATAC* mutaties), één patiënt met agammaglobulinemie (door een *BTK* mutatie), en drie patiënten met een voorafgaande diagnose van IPH of CVID (door *GATA2*, *NFKB1*, of *IKZF1* mutaties). In vijf van de zeven genetisch gediagnosticeerde patiënten hebben karakteristieke (extra-)immunologische kenmerken uiteindelijk doorslaggevende aanwijzingen gegeven. Opvallend was dat doelgerichte sequenering van gekende ziektegenen maar een beperkte opbrengst had van 5.7% (4/70). WES onthulde nieuwe mutaties in gekende ziektegenen in twee van de 13 geteste index patiënten (15%), gekende ziekte-beïnvloedende varianten in vier patiënten (31%), en nieuwe kandidaat ziektegenen in drie patiënten (23%). De genetische analyses in deze cohorte zijn nog lopend.

In **hoofdstuk 5** bestudeerden we een familie met een nieuwe heterozygote mutatie in *KMT2A* die Wiedemann-Steiner syndroom (WSS) veroorzaakt, en een tweede familie met zeldzame, samengesteld heterozygote mutaties in het niet-coderende RNA gen *RNU4ATAC* die Roifman syndroom (RS) veroorzaken. In beide families presenteerden de broer/zus duo's zich uitzonderlijk met een CVID fenotype in de vroege kinderjaren terwijl extra-immunologische kenmerken minder duidelijk waren op dat moment, wat het vinden van de juiste diagnose aanzienlijk heeft vertraagd. Dit onderlijnt het belang van het nastreven van een genetische diagnose in patiënten met een vroeg CVID fenotype. Daarnaast vonden we niet eerder gerapporteerde afwijkingen in circulerende folliculaire T helper (cTfh) cellen in WSS en RS, alsook in B cel BAFF-R expressie in RS, die de basis kunnen vormen voor nieuwe pathofysiologische inzichten in deze syndromale aandoeningen.

Vervolgens werd in twee patiënten en hun moeder een nieuwe, heterozygote mutatie gevonden in *IKZF1* dat resulteerde in IKAROS haploinsufficiëntie (**hoofdstuk 6**). De drie studiepersonen hadden allen een variabele, multi-level centrale B cel ontwikkelingsstoornis dat bovendien op een later niveau was gesitueerd in vergelijking met het ontwikkelingsstoornisniveau voordien gerapporteerd in symptomatische gevallen. Alhoewel de moeder een verminderd aantal B cellen vertoonde in het beenmerg, waren haar B cel subsets in het perifere bloed normaal en ontwikkelde ze geen klinische klachten. In deze studie beschreven we nieuwe inzichten in de centrale B cel ontwikkeling in zowel symptomatische als asymptomatische *IKZF1* mutatiedragers.

Tot slot werd in **hoofdstuk 7** een nieuw kandidaat ziektegen, namelijk *GTF3A*, geïdentificeerd. Meer bepaald hebben we nieuwe, samengesteld heterozygote varianten in *GTF3A* gevonden in een Belgische broer en zus met een CVID-achtig fenotype en ernstige B cel delingsdefecten. Preliminaire functionele validatiestudies toonden aan dat de *GTF3A* varianten potentieel pathogeen zijn door verlies van functie. Aangezien *GTF3A* een gekende rol speelt in ribosoom biogenese en sommige ribosomopathieën vroeger reeds geassocieerd werden met diverse afwijkingen in lymfocyten, veronderstellen we dat biallelische mutaties in *GTF3A* leiden tot een nieuwe ribosomopathie, gekenmerkt door een CVID-achtig fenotype. Toekomstige studies zijn gepland om een direct causaal verband tussen het *GTF3A* genotype en het fenotype van de patiënten na te kijken.

Curriculum Vitae

CURRICULUM VITAE

Personalia

Name	Delfien Joke Ann Bogaert
Date of birth	17/11/1988
Place of birth	Ghent
Nationality	Belgian
Marital status	Legal cohabitation with Thierry De Meyer
Email	Delfien.Bogaert@ugent.be

Education

2013 - Present	Master in Specialist Medicine: Pediatric Medicine *Ghent University Hospital, Ghent, Belgium*
2013 - 2017	PhD in Health Sciences *Ghent University, Ghent, Belgium* *Promotors: Prof. Dr. Elfride De Baere, Prof. Dr. Filomeen Haerynck, Dr. Melissa Dullaers* *Research Foundation - Flanders (FWO) Aspirant Fellowship*
2009 - 2013	Master in Medicine: Hospital Medicine *Magna cum laude* *Ghent University, Ghent, Belgium*
2006 - 2009	Bachelor in Medicine *Summa cum laude* *Ghent University, Ghent, Belgium*
2000 - 2006	Secondary School: Science-Mathematics *Onze-Lieve-Vrouw College, Ledeberg, Belgium*

Fellowship abroad

8/2016 - 1/2017	Research Fellowship in Primary Immunodeficiencies *National Institutes of Health, Bethesda, MD, USA* *Under supervision of Dr. Sergio D. Rosenzweig*

Grants

2013	Research Foundation - Flanders (FWO) Aspirant Fellowship grant
2015	FWO Aspirant Fellowship grant renewal
2016	FWO travel grant for a long stay abroad
2016	ESID travel grant for a medium-term fellowship abroad
2016	Ghent University - Faculty of Medicine and Health Sciences - Mobility Fund travel grant for participation at an international congress

International appointment

2016 - Present	European Society for Immunodeficiencies Junior Country Representative Belgium

Publications in A1 journals

Bogaert DJ, Kuehn HS, Bonroy C, Calvo KR, Dehoorne J, Vanlander AV, De Bruyne M, Cutlak U, Bigley V, De Baets F, De Baere E, Rosenzweig SD, Haerynck F*, Dullaers M*. A novel IKAROS haploinsufficiency kindred with unexpectedly late and variable B cell maturation defects. The Journal of Allergy and Clinical Immunology. In press.

Bogaert DJ, Dullaers M, Kuehn HS, Leroy BP, Niemela JE, De Wilde H, De Schryver S, De Bruyne M, Coppieters F, Lambrecht BN, De Baets F, Rosenzweig SD, De Baere E*, Haerynck F*. Early-onset primary antibody deficiency resembling common variable immunodeficiency challenges the diagnosis of Wiedemann-Steiner and Roifman syndromes. Nature Scientific Reports. 2017;7(1):3702.

Bogaert DJ, De Bruyne M, Debacker V, Depuydt P, De Preter K, Bonroy C, Philippe J, Bordon V, Lambrecht BN, Kerre T, Cerutti A, Vermaelen KY, Haerynck F, Dullaers M. The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. Haematologica. 2017;102(1):192-202.

Van Gorp H, Saavedra PH, de Vasconcelos NM, Van Opdenbosch N, Vande Walle L, Matusiak M, Prencipe G, Insalaco A, Van Hauwermeiren F, Demon D, Bogaert DJ, Dullaers M, De Baere E, Hochepied T, Dehoorne J, Vermaelen KY, Haerynck F, De Benedetti F, Lamkanfi M. Familial Mediterranean fever mutations lift the obligatory requirement for microtubules in Pyrin inflammasome activation. Proc Natl Acad Sci USA. 2016;113(50):14384-14389.

Bogaert DJ, Dullaers M, Lambrecht BN, Vermaelen KY, De Baere E, Haerynck F. Genes associated with common variable immunodeficiency: one diagnosis to rule them all? J Med Genet. 2016;53(9):575-90. (Review)

Alves de Medeiros AK, Lodewick E, Bogaert DJ, Haerynck F, Van Daele S, Lambrecht B, Bosma S, Vanderdonckt L, Lortholary O, Migaud M, Casanova JL, Puel A, Lanternier F, Lambert J, Brochez L, Dullaers M. Chronic and invasive fungal infections in a family with CARD9 deficiency. J Clin Immunol. 2016;36(3):204-209.

Bogaert DJ, Van Schil K, Taghon T, Bordon V, Bonroy C, Dullaers M, De Baere E, Haerynck F. Persistent rotavirus diarrhea post-transplant in a novel JAK3-SCID patient after vaccination. Pediatr Allergy Immunol. 2016;27(1),93-96.

De Bruyne R, Bogaert DJ, De Ruyck N, Lambrecht BN, Van Winckel M, Gevaert P, Dullaers M. Calcineurin inhibitors dampen humoral immunity by acting directly on naive B cells. Clin Exp Immunol. 2015;180(3),542-550.

De Bruyne R, Gevaert P, Van Winckel M, De Ruyck N, Minne A, Bogaert DJ, Van Biervliet S, Vande Velde S, Smets F, Sokal E, Gottrand F, Vanhelst J, Detry B, Pilette C, Lambrecht BN, Dullaers M. Raised immunoglobulin A and circulating T follicular helper cells are linked to the development of food allergy in paediatric liver transplant patients. Clin Exp Allergy. 2015;45(6),1060-1070.

Book chapters

Bogaert DJ, Dullaers M, De Baere E, Haerynck F. Molecular genetics of common variable immunodeficiency. In: eLS. John Wiley & Sons Ltd, Chichester. ISBN: 9780470015902 (online). In press.

Oral presentations

Bogaert DJ, Haerynck F, De Baere E, Dullaers M, Vermaelen KY. Use of an integrative classification system and NGS for gene identification in common variable immunodeficiency disorder. Research Day, Faculty of Medicine and Health Sciences, Ghent University, 2014, Ghent, Belgium.

Bogaert DJ, Dullaers M, Staal J, Gerlo S, Vandermarliere E, Debacker V, Bordon V, De Waele K, Meyts I, Moens L, Bossuyt X, De Baets F, Lambrecht BN, Vermaelen KY, Haerynck F. A novel STAT1 mutation in a patient with pneumocystis jiroveci and chronic mucocutaneous candidiasis. 43th Annual Congress of the Belgian Society of Pediatrics, 2015, Liege, Belgium.

Bogaert DJ, Debacker V, Kerre T, Bordon V, Lambrecht BN, Vermaelen KY, De Baets F, De Baere E, Dullaers M, Haerynck F. CVID, isolated IgG deficiency and isolated IgG subclass deficiency: clinical features and B cell maturation of the Ghent cohort. 44th Annual Congress of the Belgian Society of Pediatrics, 2016, Brussels, Belgium.

Bogaert DJ, Dullaers M, Kuehn HS, Leroy BP, Niemela JE, De Wilde H, De Schryver S, De Bruyne M, Coppieters F, De Baets F, Rosenzweig SD, De Baere E, Haerynck F. Early-onset common variable immunodeficiency as a cardinal manifestation challenges the diagnosis of Wiedemann-Steiner and Roifman syndromes. 45th Annual Congress of the Belgian Society of Pediatrics, 2017, Antwerp, Belgium.

Bogaert DJ, De Bruyne M, Debacker V, Depuydt P, De Preter K, Bonroy C, Philippé J, Bordon V, Lambrecht BN, Kerre T, Cerutti A, Vermaelen KY, Haerynck F, Dullaers M. The immunophenotypical fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. Research Day, Faculty of Medicine and Health Sciences, Ghent University, 2017, Ghent, Belgium.

Poster presentations

Bogaert DJ*, Debacker V, Callens S, Ommeslagh D, Dullaers M, Vermaelen KY. A case of isolated cerebral nocardiosis caused by a defect in the IL12 - IFNgamma axis. 16th Biennial Meeting of the European Society for Immunodeficiencies, 2014, Prague, Czech Republic.

Bogaert DJ*, Dullaers M, Vande Walle J, Stordeur P, Govaere E, Haerynck F. Acquired partial lipodystrophy: a rare clinical presentation of a complement deficiency. 16th Biennial Meeting of the European Society for Immunodeficiencies, 2014, Prague, Czech Republic.

Haerynck F*, Bogaert DJ, De Baere E, Lambrecht BN, Vermaelen KY, Debacker V, Van Schil K, De Baets F, Schelstraete P, Bordon V, Dullaers M. Clinical presentation of hyper-IgE syndrome in a family with impaired IL-22 production and STAT3 phosphorylation. 16th Biennial Meeting of the European Society for Immunodeficiencies, 2014, Prague, Czech Republic.

Bogaert DJ*, Van Schil K, Taghon T, Bordon V, Dullaers M, De Baere E, Haerynck F. A novel Janus kinase 3 (JAK3) mutation in a patient with severe combined immunodeficiency. 43th Annual Congress of the Belgian Society of Pediatrics, 2015, Liege, Belgium.

Bogaert DJ*, Dullaers M, Vande Walle J, Stordeur P, Govaere E, Haerynck F. Acquired partial lipodystrophy: a rare clinical presentation of a complement deficiency. 43th Annual Congress of the Belgian Society of Pediatrics, 2015, Liege, Belgium.

De Bruyne R*, Van Winckel M, Gevaert P, De Ruyck N, Bogaert DJ, Lambrecht BN, Dullaers M, Smets F, Sokal E, Gottrand F, Pilette C. Raised immunoglobulin A and circulating T follicular helper cells are linked to the development of food allergy in pediatric liver transplant patients. IPTA 8th Congress on Pediatric Transplantation, 2015, San Francisco, CA, USA.

De Bruyne R*, Gevaert P, Van Winckel M, De Ruyck N, Minne A, Bogaert DJ, Van Biervliet S, Vande Velde S, Smets F, Sokal E, Gottrand F, Vanhelst J, Detry B, Pilette C, Lambrecht BN, Dullaers M, Rogiers X. Immunological Characterization of Pediatric Liver Transplant Associated Food Allergy. 21st Annual International Congress of the International Liver Transplant Society, 2015, Chicago, IL, USA.

Bogaert DJ*, De Bruyne M, Debacker V, Depuydt P, De Preter K, Bonroy C, Philippe J, Bordon V, Lambrecht BN, Kerre T, Cerutti A, Vermaelen KY, Haerynck F, Dullaers M. The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. 17th Biennial Meeting of the European Society for Immunodeficiencies, 2016, Barcelona, Spain.

Hoste L, De Bruyne M*, Bogaert DJ, De Baere E, Bonroy C, Schelstraete P, Dullaers M, Vercruysse T, Haerynck F. Recurrent cutaneous abscesses as the presenting manifestation of STAT1 gain-of-function mutation. 17th Biennial Meeting of the European Society for Immunodeficiencies, 2016, Barcelona, Spain.

Haerynck F*, Verloo P, Bogaert DJ, Smet J, Vanlander AV, Bordon V, Verhelst H, Van Coster R, Menten B, Dullaers M. RC3H1 mutation with increased ICOS expression causes an autoinflammatory syndrome. 17th Biennial Meeting of the European Society for Immunodeficiencies, 2016, Barcelona, Spain.

Haerynck F, Bogaert DJ, De Baere E, Van Schil K, Coppieters F, De Bruyne M, Hagendorens M, Kerre T, De Baets F, Bordon V, Vermaelen KY, Tavernier J, Lambrecht BN, Gerlo S, Dullaers M*. JAK2 deficiency as a novel cause of impaired Th17 immunity. 17th Biennial Meeting of the European Society for Immunodeficiencies, 2016, Barcelona, Spain.

Bogaert DJ*, De Bruyne M, Debacker V, Depuydt P, De Preter K, Bonroy C, Philippe J, Bordon V, Lambrecht BN, Kerre T, Cerutti A, Vermaelen KY, Haerynck F, Dullaers M. The immunophenotypic fingerprint of patients with primary antibody deficiencies is partially present in their asymptomatic first-degree relatives. 17th Biennial Meeting of the European Society for Immunodeficiencies, 2016, Barcelona, Spain.

Bogaert DJ*, Kuehn HS, Bonroy C, Calvo KR, Dehoorne J, Vanlander AV, De Bruyne M, Cutlak U, Bigley V, De Baets F, De Baere E, Rosenzweig SD, Haerynck F, Dullaers M. To B or not to B matured: a novel IKAROS haploinsufficiency kindred with unexpectedly late and variable B cell maturation defects. 1st Focused Meeting of the European Society for Immunodeficiencies: “Autoimmunity and Inflammation in Primary Immunodeficiency; beyond the paradox”, 2017, Edinburgh, UK.

De Bruyne M*, Hoste L, Bogaert DJ, Migaud M, Konopnicki D, Lambrecht B, Beyaert R, De Baere E, Puel A, Goffard JC, Haerynck F, Staal J, Dullaers M. A CARD9 Turkish founder mutation disrupts NF-κB signaling by inhibiting Bcl10 recruitment. 1st Focused Meeting of the European Society for Immunodeficiencies: “Autoimmunity and Inflammation in Primary Immunodeficiency; beyond the paradox”, 2017, Edinburgh, UK.

Additional courses and workshops

2009	13th SCMSA summer school on infectious diseases and tropical medicine. Ismailia, Egypt.
2010	17th International summer school on pediatrics. Groningen, The Netherlands.
2010	Basics of electrocardiography. Ghent, Belgium
2011	1st International summer school on health & migration. Ghent, Belgium.
2013	Clinical studies: study design, implementation and reporting. Ghent, Belgium
2013	Optimized solutions for multicolor experiments by flow cytometry. BD Biosciences, Erembodegem, Belgium
2014	Advanced academic English writing skills. Ghent, Belgium
2015	European Society for Immunodeficiencies summer school. Calambrone, Italy
2016	Analyzing flow cytometry data using FlowJo. VIB, Ghent, Belgium.
2016	Communication skills: basic course, conflict management. Ghent, Belgium
2016	Genetic engineering. VIB, Ghent, Belgium.
2017	4th Workshop on diagnostics of immunodeficiencies. Freiburg-Munzingen, Germany.

Supervision of master students

Master thesis, 1st and 2nd Master in Medicine 2015-2017. De Craemer Jarno and De Ketelaere Laurens. Geïsoleerde immunoglobuline G (IgG) subklasse deficiëntie bij kinderen en volwassenen: immunologisch fenotype en nood aan behandeling?
Promotor: Prof. Dr. Tessa Kerre. Co-promotor: Prof. Dr. Filomeen Haerynck. Supervision: Delfien Bogaert.

Dankwoord

DANKWOORD

Hier is het dan, mijn doctoraatsthesis. De voorbije vier jaar kan ik het best omschrijven met de woorden van Walt Disney: “We keep moving forward, opening new doors, and doing new things, because we're curious and curiosity keeps leading us down new paths.” Wetenschappelijke nieuwsgierigheid was dan wel de onderliggende drijfveer van mijn doctoraat, dit werk zou niet tot stand gebracht zijn zonder de bijdrage van talrijke mensen.

In de eerste plaats wil ik mijn drie promotoren bedanken. Jullie hebben me voortreffelijk en vol vertrouwen begeleid. Elfride, bedankt om het hoofdpromotorschap op jou te nemen en me de mogelijkheid te geven om samen te werken met jouw team binnen het Centrum Medische Genetica Gent (CMGG). Ondanks je overvolle agenda vond je toch de tijd om me te begeleiden bij het genetische luik van mijn onderzoek. Jouw kalmte, optimisme en gedrevenheid werkten steeds bemoedigend. Filomeen, tijdens mijn co-assistentschap pediatrie bracht je me in contact met de wondere wereld van PID. Het duurde dan ook niet lang voordat ik vroeg of ik zou kunnen doctoreren op dit onderwerp. In de daaropvolgende jaren heb je me heel wat kansen geboden, waaronder mijn introductie in de ESID (European Society for Immunodeficiencies) gemeenschap en de mogelijkheid om zes maanden onderzoek te verrichten aan de prestigieuze National Institutes of Health (NIH) in de VS. Verder kon ik steeds op je rekenen om me de klinische knepen van het vak bij te brengen en kritisch feedback te geven op mijn onderzoekswerk. Ik bewonder jouw onuitputtelijk enthousiasme, jouw toewijding aan je patiënten en jouw doorzettingsvermogen in wetenschappelijke projecten. Melissa, vooreerst ben ik je heel dankbaar dat je vier jaar geleden een FWO project voor mij hebt uitgeschreven. Het verkrijgen van deze onderzoeksbeurs was immers het startschot van mijn doctoraatstraject. Daarnaast heb je mij niet alleen begeleid bij het immunologische luik van mijn doctoraat, maar ook bij elke stap van elke weg die ik insloeg, met veel geduld en ruimte om zelf te ontdekken. Je leerde me denken en werken als een wetenschappelijk onderzoeker. Bedankt voor jouw betrokkenheid, jouw wetenschappelijke blik en de ongedwongen sfeer in onze bureau.

I wish to express my gratitude to the members of the examination committee, Prof. Dr. Michel Moutschen, Prof. Dr. Hamida Hammad, Prof. Dr. Rudi Beyaert, Prof. Dr. Petra Schelstraete and Dr. Frauke Coppieters, for carefully reading and evaluating my thesis. I greatly appreciated our discussions during the internal defense. Prof. dr. Johan Vande Walle, thank you for taking on the role of chair of the examination committee.

Prof. Em. Dr. Dirk Matthys, voormalig diensthoofd pediatrie, en Prof. Dr. Sabine Van daele, huidig diensthoofd pediatrie, hartelijk dank voor de mogelijkheid om mijn opleiding pediatrie te combineren met een doctoraat en om me de vrijheid te geven om me vier jaar lang te verdiepen in fundamenteel wetenschappelijk onderzoek binnen PID.

De collega's van het Tumor Immunology Lab/Clinical Immunology Research Lab wil ik bedanken voor alle hulp en steun tijdens de afgelopen jaren. Karim, bedankt om mij een vaste stek te geven in jouw onderzoekslab en voor de fijne samenwerking tijdens de eerste jaren van mijn doctoraat. Veronique, Nancy en Kelly, ik wil jullie uitdrukkelijk bedanken voor de hulp bij het verwerken van de vracht aan bloedstalen. Ook voor andere zaken kon ik op jullie rekenen. Veronique, bedankt voor jouw bijdrage bij het recruteren van (minderjarige) gezonde controles en het uitvoeren van flow cytometrie kleuringen. Nancy, dank voor de ondersteuning bij het moleculair genetisch werk. Kelly, bedankt voor de stipte opvolging van de bestellingen. Ex-bureaugenoten Lotte en Elisabeth, onze wegen zijn intussen gescheiden, maar bedankt voor de praktische hulp bij mijn eerste stappen in het labo in het "oude" MRB gebouw. Huidige bureaugenoot Lien, bedankt voor de fijne babbels tijdens de laatste maanden van mijn doctoraat. Simon, jij hebt pas recent het PID team versterkt. Het was een plezier om met je samen te werken en ik wens je alle succes toe met jouw onderzoeksprojecten en specialisatie.

Veel dank aan de collega's van het CMGG voor hun ondersteuning en hulp bij het moleculair genetisch werk. Kristof, dank je voor jouw deskundige uitleg, begeleiding en enthousiasme bij mijn eerste stappen in het sequeneren van genen. Het allerbeste met je nieuwe postdoc. Marieke, bedankt voor alle praktische hulp bij whole exome sequencing, screenen van genen, enz. Jij was vaak een onmisbare schakel tussen mij en het team in het CMGG, maar je was bovenal een geweldige collega. Veel succes met je doctoraat. Thalia, bedankt voor het lopen van de vele exomen. Toon wil ik bedanken voor het lopen van de scripts voor WES analyse. Pauline, dank je voor de hulp bij de "unsupervised computational clustering analysis" van de FACS data. Tot slot wil ik de medewerkers bedanken van het secretariaat, het DNA extractie team, het FORD team, de GSU, en alle anderen die op een of andere manier hebben bijgedragen aan dit werk.

Bart Lambrecht, bedankt dat ik mijn onderzoekswerk mocht voorstellen op jullie labmeetings en voor jouw kritische inbreng. Daarnaast ben ik je ook dankbaar dat ik de seminaries van het VIB Center for Inflammation Research mocht bijwonen. Gert Van Isterdael, dank je voor jouw advies bij de optimalisatie van de flow cytometrie kleuringen.

My thesis would not have been what it is today without my research stay at the NIH. Sergio and Tom, I am deeply grateful that you have given me the opportunity to work in your lab for six months, and for your knowledge and guidance during that time. Hyesun, I cannot thank you enough for all your help, support and guidance. It has been an honor working with such a talented and passionate scientist. Julie, thank you for your help in re-analyzing our WES data and for introducing me to the world of computational protein modeling. I really enjoyed our pleasant conversations. Cecilia, Jahnavi, Jen, Kim, Jude, Shakuntala, Chung-Hee, Gina, Heather, Kathy, Gabby and Genieva, thank you all for making my time in the US so memorable. Thank you for the warm welcome, the nice chats, the Halloween events such as candy stick racing, the “lunch parties” and the delicious cakes every week.

Ik wil graag alle artsen, verpleegkundigen en logistieke en administratieve medewerkers bedanken die bijgedragen hebben aan het recruteren van patiënten, registreren van klinische informatie en/of afnemen van bloedstalen. In het bijzonder gaat mijn dank uit naar de medewerkers en verpleegkundigen van de poli pediatrie alsook Frans De Baets, Myriam Van Winckel, Ruth De Bruyne, Victoria Bordon, Joke Dehoorne, Bart Leroy en Bert Callewaert. Tessa, een groot deel van de volwassen patiënten zou er niet geweest zijn zonder jouw hulp. Bedankt om je patiënten te motiveren om deel te nemen aan mijn studie, maar bovenal bedankt voor de heel prettige samenwerking.
Dank aan het team van de Klinische Biologie, onder leiding van Jan Philippé en Carolien Bonroy. De analyses die jullie dagelijks in routineverband uitvoeren zijn onmisbaar bij de immunologische uitwerking van onze patiënten. Daarenboven zijn jullie ook steeds bereid om jullie expertise en kunde in te zetten voor wetenschappelijk onderzoek.
Mijn dank gaat eveneens uit naar Linda Reunis en de medewerkers van het secretariaat kinderpneumologie en het algemeen secretariaat pediatrie voor de administratieve ondersteuning. Bart Coucquyt, dank je voor de uitstekende opvolging van alle financiële aspecten van mijn doctoraat.

Mijn oprechte dank aan alle patiënten en hun families die deelnamen aan mijn studies en die, ondanks hun zorgen, bereid waren om toch nog een extra keer naar het ziekenhuis te komen of een extra bloedafname te laten doen. Eveneens hartelijk dank aan alle gezonde vrijwilligers voor het doneren van bloed. Het zien van de bereidwillige medewerking van al deze mensen deed me steeds opnieuw beseffen waarvoor ik het deed.

Vrienden en familie, bedankt voor jullie onophoudelijke steun, de bemoedigende woorden als het wat moeilijker ging en de nodige verstrooiing. Jullie gaven me de energie om verder te gaan. Christophe en Silke, en sinds kort ook Ellie, bedankt voor de gezellige etentjes en

filmavonden. Nienke, Maxim, kleine Vic, Vicky, Malik, Heidi, Bram en Lynn, bedankt voor de leuke “get-togethers”. Bert en Jolien, bedankt voor jullie warme vriendschap ook al is het niet eenvoudig om een gaatje te vinden in onze drukke agenda’s.
Dank aan mijn peter Johan, Christine, Dimitri, Marie, mijn broers David en Michaël, mijn schoonzussen Marieke en Marijke, en mijn nichtje Nora. Familiefeestjes zijn steeds een gezellige drukte waar ik naar uitkijk. Meter Agnes, Paul, Heidi, Richard en Gina, bedankt dat jullie na al die jaren nog steeds vol interesse vragen hoe het gaat met mijn studies. Marc en Lutgarde, mijn “schoonouders”, bedankt voor alles dat jullie voor ons gedaan hebben de voorbije jaren en om bij te springen als dat nodig is.

Een speciaal woord van dank aan mijn grootmoeder Jacqueline en mijn ouders. Jullie hebben me gemaakt tot wie ik ben vandaag. Hard werken, doorzettingsvermogen en gedrevenheid zijn waarden die ik zonder twijfel van jullie heb meegekregen. Mama en papa, “dank je” lijkt niet voldoende om uit te drukken hoe dankbaar ik jullie ben. Jullie hebben me steeds aangemoedigd en gesteund in al mijn jaren van studeren en doctoreren. Jullie staan altijd klaar met een helpende hand of advies, niets is jullie te veel gevraagd. Dat jullie mijn rots in de branding zijn ervaar ik dan ook als heel bijzonder.

Liefste Thierry, bedankt voor jouw onvoorwaardelijke steun, zorg en liefde. Bedankt om me te steunen in mijn ambities en voor jouw begrip op momenten dat ik afwezig ben. Ik ben heel dankbaar voor jou in mijn leven en voor alle mooie avonturen die we de voorbije jaren samen hebben beleefd. Off to another adventure!